Remarks

Reconsideration of this Application is respectfully requested.

Claims 23-35 and 37-78 are pending in the application, with 23, 40, 51, 66, 75 and 77 being the independent claims. Claims 23-35 and 37-74 are rejected.

The specification has been amended merely to update the status of a related application. This change is believed to introduce no new matter, and its entry is respectfully requested.

Based on the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding rejections and that they be withdrawn.

I. Election / Restriction

The Examiner acknowledged Applicants' election with traverse of Group I, claims 23-74. The Examiner also acknowledged that the process claims of Group II, claims 75-78, should be rejoined if the pending product claims of Group I, claims 23-25 and 36-74, are found allowable. (*See* Office Action, page 2.)

II. Rejections under 35 U.S.C. § 101

The Examiner maintained the rejection of claims 23-35 and 37-74 under 35 U.S.C. § 101 as allegedly not being supported by either a specific and substantial utility or a well established utility. (*See* Office Action, page 3.) In particular, it is the Examiner's position that

> the instant invention lacks a specific and substantial real world utility absent elucidation of the biological function of the disclosed protein agonist which the claimed antibody is directed and any role that the antibodies identified as modulators of the protein would play in modulation or identification of any disease state associated with that biological function.

(Office Action, page 4.) Applicants respectfully disagree with the Examiner's rejection. Applicants have in fact disclosed a biological function of the EBI-2 G-protein coupled receptor as well as a role the claimed antibodies would play in modulation of a specific disease state.

A. The Specification Discloses at Least One Specific Biological Function of EBI-2 G Protein-Coupled Receptors which is Substantial.

The Examiner asserted that "no disclosure is provided within the instant specification as to any specific biological function of the polypeptide having SEQ ID NO:2 or any specific disease where the claimed invention could be used." (Office Action, page 5.)

The Utility Guidelines define "specific utility" as a utility that

> is *specific* to the subject matter claimed. This contrasts with a *general* utility that would be applicable to the broad class of the invention. . . . For example, indicating that a compound may be useful in treating unspecified disorders, or that the compound has "useful biological" properties, would not be sufficient to define a specific utility for the compound. . . . Contrast the situation where an applicant discloses a specific biological activity and reasonably correlates that activity to a disease condition. Assertions falling within the latter category are sufficient to identify a specific utility for the invention.

MPEP § 2107.01 at 2100-32.

Applicants respectfully emphasize that the specification does NOT assert that the claimed invention "may be useful in treating unspecified disorders." The specification clearly and unambiguously asserts that: (1) EBI-2 polypeptide is a G protein-coupled receptor (*see* specification, ¶ [0034]); (2) such "G protein-coupled receptor molecules and their associated G-proteins have been implicated in . . . adenylyl cyclase signal channels" (specification, ¶ [0035]); (3) "[p]otential therapeutic and/or diagnostic applications for such a factor may include . . . heart disease . . . atherosclerosis, restenosis . . . ." (*id.*); and (4) antagonists for such receptors can be used to treat "hypertension, angina pectoris, myocardial infarction...." (specification, ¶ [0097]).

Clearly the specification discloses "a specific biological activity," *i.e.,* adenylyl cyclase signaling activity and "reasonably correlate[d] that activity to a disease condition," *i.e.,* heart disease or myocardial infarction, as opposed to "unspecified disorders." The asserted utility in the specification therefore falls "within the latter category [that] is sufficient to identify a specific utility for the invention." *See* MPEP ¶2107.01 at 2100-32.

B. The Assertion of Utility for EBI-2 Receptors is Reasonable in View of the Information Available at the Time of Filing.

Relying on *Brenner v. Manson*, 383 U.S. 519 (1966), it is the Examiner's position that the asserted utility is speculative in view of sequence homology alone. (*See* Office Action, pages 4-5.) Specifically, the Examiner asserted that

> [t]he specification discloses that the polypeptide of amino acids 1-342 or 2-342 of SEQ ID NO:2 has about 25% identity and 49% similarity to the EBI-1 gene over an approximately 350 amino acid stretch. . . . Both EBI-1 and

> EDG-1 are found in a variety of tissue and are themselves considered orphan receptors.
>
> . . . Speculating a function of a protein merely based on that EBI-2 is a GPCR and would have some use in research and development is not asserted utility.

(Office Action, pages 4-5.)

Applicants' assertion of utility is more than mere speculation. It is evident from the record that Applicants have provided *more* than just homology information in support of the assertions of utility for the claimed invention. Further, "Office personnel should be careful . . . not to label certain types of inventions as . . . 'speculative' as such labels do not provide the correct focus for the evaluation of an assertion of utility." MPEP §2107.02 at 2100-40.

Contrary to what the Examiner suggests, Applicants need not *confirm* any asserted utility. Applicants need only to *assert* one specific utility of the claimed invention; all aspects of the claimed utility are not required to be proven. *See Nelson v. Bowler*, 626 F.2d 853, 856-57 (CPPA 1980). An assertion of utility need only be "reasonably predictive" (as opposed to "reasonably confirmed"); it need not be a "statistical certainty." *See, e.g., Rey-Bellet v. Englehardt*, 493 F.2d 1380 (CPPA 1974); MPEP § 2107.01; *Nelson v. Bowler*, 626 F.2d 853, 856-57 (CPPA 1980). The standard is whether one skilled in the art would reasonably conclude that the asserted utility is more likely than not true. Based on the information available to one skilled in the art at the time of filing and the data presented in the specification, a person of ordinary skill in the art could reasonably conclude that antibodies to EBI-2 G protein-coupled receptors would have utility in treating heart disease, in particular, myocardial infarction.

The G protein-coupled receptor encoded by SEQ ID NO:1 is closely related to certain G protein-coupled receptors known to be induced upon Epstein-Barr virus (EBV) infection. (*See* specification, ¶ [0003].) These related EBV-induced (EBI) receptors are primarily expressed in lymphoid tissues and are related to thrombin G protein-coupled receptors. *See* Birkenbach *et al.*, *J. Virol.* 67:2209-2220 (1993) (submitted with the Amendment and Reply Under 37 C.F.R. §1.111, filed March 23, 2005.) Therefore, it was reasonable, at the time of filing, to assert that the G protein-coupled receptor encoded by SEQ ID NO:1 is related to thrombin G protein-coupled receptors.

Thrombin G protein-coupled receptors were well known as of the effective filing date of the present application. In particular, thrombin G protein-coupled receptors were known to activate platelet aggregation via adenylyl cyclase. *See* Brass *et al.*, *Thromb. and Haemost.* 70:217-223 (1993) (Exhibit A); *see also* Ohlmann *et al.*, *Biochem. J.* 312:775-779 (1995) (Exhibit B). Furthermore, thrombin G protein-coupled receptors were known to interact with G_i protein, a known inhibitor of adenylyl cyclase. *See id*; *see also* Williams *et al.*, *Blood* 76: 721-730 (1990) (Exhibit C). Since adenylyl cyclase also induces the formation of cyclic AMP (cAMP), inhibition of adenylyl cyclase both decreases cAMP formation and activates platelet aggregation. *See id.* "Most platelet agonists suppress cAMP formation by inhibiting adenylyl cyclase." *Id.* It was further known at the time that such depletion of cAMP activates platelet aggregation. *See id.*

Therefore, an artisan of ordinary skill could have *reasonably* predicted as of the filing date that the polypeptides encoded by SEQ ID NO:1 would have a function in platelet aggregation based on the information disclosed in the specification and the information available in the art. Applicants note that the specification need *not* disclose

information that was well known at the time of filing an application. *See Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 231 USPQ 81 (Fed. Cir. 1986)("A patent need not teach, and preferably omit, what is well known in the art."). In further support of the reasonableness of the asserted utility of antibodies to EBI-2 polypeptides, Hollopeter *et al.* (submitted with the Amendment and Reply Under 37 C.F.R. §1.111 filed May 6, 2003), *confirms* that the EBI-2 receptor coupled with G_i protein inhibits adenylyl cyclase and activates platelet aggregation. *See Nature* 409:202-7 (2001).

Additionally, antagonistic drugs such as clopidogrel which block platelet activation were well known as effective preventatives of vascular ischemic disease such as myocardial infarction. *See, e.g.* Schafer, *Am. J. Med.* 101:199-209 (1996) (Exhibit D). For example, Roald *et al.* found that clopidogrel acting as an antithrombotic agent inhibited thrombogenesis when human blood was tested in an *ex vivo* model. *Thromb. Haemost.* 71:655-662 (1994) (Exhibit E). Based on the pre-filing date studies, it was reasonable to assert that an antagonist to a G protein-coupled receptor involved in a pathway of platelet aggregation would block the pathway, thereby preventing platelet aggregation and consequently myocardial infarction.

As even further evidence of the reasonableness of the asserted utility, the post-filing date publication by Hebert *et al.* confirms that EBI-2 G protein-coupled receptor is indeed the target of clopidogrel. *See* Herbert *et al.*, *Sem. In Vascul. Med.* 3:113-121 (2003) (Exhibit F). Herbert *et al.* demonstrated that the active metabolite of clopidogrel covalently binds to the EBI-2 receptor and blocks the ligand binding to the EBI-2 receptor, thereby elucidating the mechanism by which clopidogrel inhibits the

aggregation of platelets. *See id.* The claimed antibodies have a similar function as clopidogrel, *i.e.*, blocking EBI-2 receptors and inhibiting platelet aggregation.

Applicants respectfully remind the Examiner that all the information showing the reasonableness of the asserted utility of EBI-2 receptors was available *before* the effective filing date of the present application; the post-filing date publications merely *confirm* the reasonableness of the asserted utility. Based on the information available at the time of filing in conjunction with the data presented by Applicants in the specification, it was *reasonable* for a person of ordinary skill in the art to assert that antibodies to EBI-2 G protein-coupled receptor would be useful for treating myocardial infarction. Considering the information that Applicants possessed and the information available at the time of filing, Applicants have clearly satisfied the "reasonably predictive" standard. *See, e.g., Rey-Bellet v. Engelhart*, 493 F.2d 1380 (CCPA 1974); MPEP §2107.01.

C. The Asserted Utility Is Independent of Knowledge of the Ligand.

Applicants also wish to point out to the Examiner that it is irrelevant to the reasonableness of the specific and substantial asserted utility whether or not the ligand for EBI-2 was disclosed. Contrary to what the Examiner suggests, an artisan of ordinary skill need not know the identity of a receptor's ligand in order to make useful antibodies against that receptor. One can block a receptor on a cell surface with an antibody to exert an inhibitory effect without knowing a ligand to the receptor, thereby interfering with the signal transduction pathway.

Applicants identified the EBI-2 G protein-coupled receptor and its effector, *i.e.*, adenylyl cyclase. (*See* specification, ¶ [0035].) Possessing both the knowledge and function of the effector downstream of the EBI-2 G protein-coupled receptor pathway, one can reasonably predict that antibodies against the EBI-2 receptor could inhibit the downstream signal transduction.

Moreover, it was well known to one of ordinary skill in the art at the time of filing that one could utilize an antibody to block a pathway of an receptor absent knowledge of the ligand. For example, Klapper *et al.* used a monoclonal antibody raised against ERB-2, an orphan receptor that belongs to a family of tyrosine kinase receptors for either epidermal growth factor (EGF) or Neu differentiation factor (NDF/neuregulin), to inhibit tumor growth. *See* Klapper *et al.*, *Oncogene* 14:2099-109 (1997) (Exhibit G). Thus, it is clear that knowledge of the ligand for a receptor is not required for antibodies to that receptor to be useful.

D. Applicants Need Only Make One Credible Assertion of Specific Utility.

The Examiner further asserted that

> The specification . . . discloses that "antagonists have been employed for treatment of hypertension, angina pectoris, myocardial infarction, ulcers, asthma, allergies, psychoses, depression, migraine, vomiting, stroke, eating disorders, migraine headaches, cancer and benign prostatic hypertrophy." However, the instant specification does not disclose that EBI-2 is associated with a particular disease.

(Office Action, page 5.)

Applicants point out that they "need only make *one* credible assertion of specific utility for the claimed invention to satisfy 35 U.S.C. 101 and 35 U.S.C. 112; additional

statements of utility, even if not 'credible,' do not render the claimed invention lacking in utility." MPEP § 2107.02 at 2100-37 (emphasis added); *see also In re Gottlieb*, 140 USPQ 665, 668 (CCPA 1964)("Having found that the antibiotic is useful for some purpose, it becomes unnecessary to decide whether it is in fact useful for the other purposes 'indicated' in the specification as possibly useful."). In fact, the Federal Circuit has indicated that

> [t]o meet the utility requirement, the Supreme Court has held that a new product or process must be shown to be "operable" - that is, it must be "capable of being used to effect the object proposed." Our cases have not, however, interpreted this language . . . to mean that a patented device must accomplish *all* objectives stated in the specification. On the contrary, "[w]hen a properly claimed invention meets at least one stated objective, utility under § 101 is clearly shown."

Carl Zeiss Stiftung v. Renishaw plc, 20 USPQ2d 1094, 1100 (Fed. Cir. 1991) (citations omitted) (quoting *Raytheon Co. v. Roper Corp.*, 220 USPQ 592, 598 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 835 (1984)).

Applicants have disclosed a specific biological activity, *i.e.,* adenylyl cyclase signaling activity, and reasonably correlated that activity to a disease condition, *i.e.,* myocardial infarction. It is therefore unnecessary to decide whether the claimed antibodies are in fact useful for the other purposes indicated in the specification to satisfy the utility requirement for the claimed invention.

E. The Use of Post-filing Date References to Substantiate Assertions of Utility is Proper.

The Examiner further asserted that:

> Hollopeter et al. (2001) and Dorsham et al. (2004) . . . describe a role of EBI-2 GPCR in platelet aggregation. Both publications . . . are after the filing date and Applicants have failed to disclose an asserted, substantial and specific utility of their invention in the specification at the time of filing the instant application.

(Office Action, page 6.)

Applicants respectfully disagree with the Examiner. Applicants established the reasonableness of the asserted utility of the claimed invention at the time of filing the application. *See e.g.* Birkenbach *et al.*, *J. Virol.* 67:2209-2220 (1993); Brass *et al.*, *Thromb. Haemost.* 70:217-223 (1993); Schafer, *Am. J. Med.* 101:199-209 (1996); and Roald *et al.*, *Thromb. Haemost.* 71:655-662 (1994). The post-filing date articles are being used to *substantiate* one of the utilities specifically asserted in the specification. Moreover, the post-filing date references *confirm* that the assertion of utility based on the knowledge available in the art at the time of filing and Applicants' disclosure was in fact reasonable and correct. They have been submitted as evidence only *after* the Examiner challenged the presumptively true assertion of utility *already in the specification.* Such submissions are expressly sanctioned by both the Federal Circuit and the PTO. *See In re Brana*, 51 F.3d 1560, 1567 n.19 (Fed. Cir. 1995); MPEP § 2107.02.

Thus, the use of the post-filing date articles and the data provided therein to corroborate assertions of utility with respect to the present invention by Applicants is proper. Therefore, Applicants respectfully assert that the remarks and arguments relating to post-filing date references in response to the utility rejection made in the previously filed Amendment and Replies (filed March 23, 2005 and May 6, 2003), are fully applicable and are incorporated by reference herein.

Applicants have asserted that the claimed antibodies can be used, for example, for the treatment of heart diseases such as myocardial infarction. In view of the facts and relationships set out above, Applicants submit that this assertion is not only specific and substantial, but credible as well, *i.e.,* the assertion is *at least believable* to, and would not be considered *false* by, a person of ordinary skill in the art. *See* MPEP § 2107.02 at 2100-40. Since the presently claimed invention possesses a credible, specific and substantial utility that constitutes a patentable utility under 35 U.S.C. § 101, Applicants respectfully request that the Examiner reconsider and withdraw the rejection of the claims under 35 U.S.C. § 101.

III. Rejections under 35 U.S.C. § 112, First Paragraph, Enablement

The Examiner maintained the rejection of claims 23-35 and 37-74 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement. (S*ee* Office Action, page 7.) For the reasons discussed above in reply to the rejections under 35 U.S.C. § 101, Applicants assert that the claimed invention complies with current case law and is supported by a specific, substantial and credible utility. The Examiner "should not impose a 35 U.S.C. § 112, first paragraph, rejection grounded on a 'lack of utility' basis unless a 35 U.S.C. § 101 rejection is proper." MPEP § 2107.01 at 2100-36. Therefore, since the claimed invention complies with the utility requirement of 35 U.S.C. § 101, the rejection under 35 U.S.C. § 112, first paragraph, based on the alleged lack of utility of the claimed invention, should be withdrawn.

IV. Rejections under 35 U.S.C. § 112, Second Paragraph

The Examiner has withdrawn the rejection of claims 29 and 30 under 35 § U.S.C. §112, second paragraph, in view of Applicants' arguments and/or amendments. (*See* Office Action, page 7.) Applicants thank the Examiner for the withdrawal of these rejections.

V. Rejections under 35 U.S.C. § 112, First Paragraph, Written Description

The Examiner has withdrawn the rejection of claim 36 under 35 § U.S.C. §112, first paragraph, for an alleged lack of written description. (*See* Office Action, page 7-8.) Applicants thank the Examiner for the withdrawal of the rejection.

VI. Rejections under 35 U.S.C. § 112, First Paragraph, Enablement

The Examiner has withdrawn the rejection of claim 36 under 35 U.S.C. §112, first paragraph, for an alleged lack of enablement. (*See* Office Action, page 8.) Applicants thank the Examiner for the withdrawal of the rejection.

Conclusion

All of the stated grounds of rejection have been properly traversed, accommodated or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Signaling through G Proteins and G Protein-coupled Receptors during Platelet Activation

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Summary

Recent studies have helped to define the early events of signal transduction in platelets. The best-described of these events are those in which heterotrimeric guanine nucleotide binding regulatory proteins (G proteins) mediate the interaction between cell surface receptors for agonists and intracellular second messenger generating enzymes. To date nine different G proteins have been identified in platelets. Their targets include phospholipases C and A_2, and adenylyl cyclase. Efforts to clone the receptors that can couple to these G proteins have been successful for epinephrine, thrombin, TxA_2 and platelet activating factor. Each of these is comprised of a

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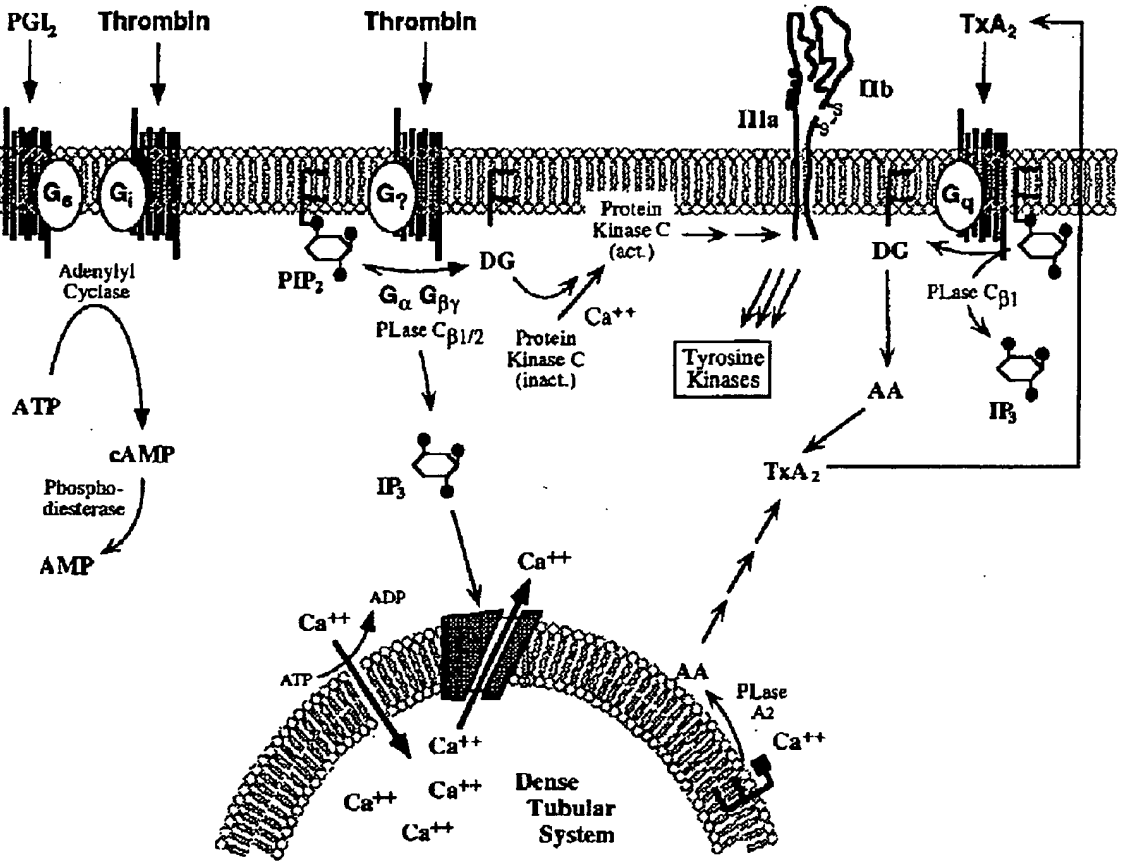

Figure 1: Signal transduction during platelet activation. The binding of agonists to receptors on the platelet surface initiates cascades of intracellular second messengers, including inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). IP_3 releases Ca^{++} from the platelet dense tubular system, raising the cytosolic free Ca^{++} concentration. Diacylglycerol activates protein kinase C, shifting it to the plasma membrane and triggering granule secretion and fibrinogen receptor exposure on the glycoprotein IIb-IIIa complex. At the same time, the rising cytosolic free Ca^{++} concentration facilitates arachidonate (AA) formation by phospholipase A_2, a process that may occur at both the plasma membrane and the dense tubular system membrane. Arachidonate is metabolized to thromboxane A_2 (TxA_2), which diffuses out of the cell, interacts with receptors on the platelet surface and causes further platelet activation. During this process tyrosine kinases, including members of the src family, are activated and phosphorylate multiple platelet proteins, most of which have not been identified. In platelets, tyrosine kinase activation appears to occur predominantly "downstream" from fibrinogen receptor expression and platelet aggregation. In many cases, the interaction between agonists and the enzymes responsible for second messenger generation is mediated by a G protein. In platelets, G proteins have been shown to regulate phosphoinositide hydrolysis and cAMP formation, and are probably involved in the activation of phospholipase A_2 as well. Phospholipase Cβ is activated in a pertussis toxin-sensitive manner by a still-unidentified G protein and in a pertussis toxin-resistant manner by G_q and/or G_{11}. Recent evidence suggests that the various forms of phospholipase Cβ differ in their regulation by G proteins, β1 being activated by G_α, β2 by $G_{\beta\gamma}$. Adenylyl cyclase is stimulated by the G protein G_s, and inhibited by the G protein G_i. The G protein that regulates phospholipase A_2 activity remains to be identified.

single polypeptide with seven transmembrane domains and an extracellular N-terminus. In the case of the thrombin receptor, activation occurs by a novel mechanism in which thrombin cleaves its receptor, creating a new N-terminus that can serve as a tethered ligand. Shortly after activation, thrombin receptors become resistant to re-activation by thrombin. This desensitization, which appears to involve receptor phosphorylation and internalization, provides a potent mechanism for limiting the duration of thrombin-initiated events in platelets and other thrombin-responsive vascular cells.

Platelet activation

A variety of agonists are able to activate platelets, including collagen, ADP, thromboxane A_2, epinephrine and thrombin. Collagen and thrombin serve as primary activators at sites of vascular injury, while released ADP and thromboxane A_2 help to recruit additional platelets into a growing platelet plug. According to current models, the receptors for most platelet agonists are formed by proteins which cross the plasma membrane one or more times. The extracellular and transmembrane domains of the receptor form the agonist binding site, while the cytosolic domains interact with second-messenger-producing enzymes and ion channels whose activity is modulated by receptor occupation. Often at least one additional protein is required for this process. Commonly, this protein is a member of a family of heterotrimeric GTP-binding regulatory proteins called G proteins. These proteins mediate the interaction between receptors and effectors and, depending upon the particular G protein, may either stimulate or inhibit the effector (Fig. 1).

Two intracellular pathways play a central role in platelet activation by most agonists. Each begins with the enzymatic hydrolysis of specific membrane phospholipids. The phosphoinositide pathway starts when phosphatidylinositol 4,5-bisphosphate (PIP_2) is cleaved by phospholipase C to form inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, both of which serve as second messengers in platelets (Fig. 1). IP_3 releases Ca^{++} from the platelet dense tubular system and contributes to the rise in the cytosolic free Ca^{++} concentration that typically accompanies platelet activation. In turn, this promotes the activity of enzymes which are not fully functional at the low Ca^{++} concentration present in resting platelets. Diacylglycerol activates protein kinase C, leading to protein phosphorylation on serine and threonine residues, granule secretion and fibrinogen receptor expression. A second pathway begins when arachidonate is released from membrane phospholipids either by the direct action of phospholipase A_2 or by the sequential action of phospholipase C and diacylglycerol lipase. The newly liberated arachidonate is then metabolized to thromboxane A_2 (TxA_2) which is a potent stimulus for platelet activation. Since TxA_2 can diffuse across the platelet plasma membrane, it can serve as a messenger between platelets as well as within platelets, interacting with receptors on the cell surface.

While the production of each of these second messengers promotes platelet aggregation, the formation of cyclic AMP (cAMP) inhibits platelet activation. Agents such as prostaglandin I_2 (PGI_2) which raise platelet cAMP levels dampen platelet responsiveness, presumably by activating cAMP-dependent protein kinases. Known platelet substrates for this enzyme include glycoprotein (GP) Ib_β, actin binding protein, myosin light chain and *rap*1b. However, it is not yet clear how the phosphorylation of any of these accounts for the observed inhibition of platelet activation. Most platelet agonists suppress cAMP formation by inhibiting adenylyl cyclase. Some may also accelerate the metabolism of cAMP by stimulating cAMP phosphodiesterase.

The hallmark of platelet activation is aggregation, an event that is dependent upon the successful binding of fibrinogen to the plasma membrane GP IIb-IIIa (or α_{IIb}-β_3) complex. The IIb-IIIa complex is present on the surface of resting as well as activated platelets, but undergoes a conformational transition during platelet activation that enables it to serve as a binding site for fibrinogen and other adhesive proteins. The precise nature of this transformation is still under investigation, as is the full sequence of intracellular events that triggers it.

Agonist receptors

Until recently, far more was known about platelet responses to agonists than about the structure of the receptors for those agonists. Recently, however, there has been a great deal of progress in this area, highlighted by the successful cloning of the receptors for TxA_2 (1), platelet activating factor (2), and thrombin (3,4). Each of these has proven to be a member of the G protein-coupled family of receptors, a family already known to include platelet α_2-adrenergic receptors (5). Each is formed by a single polypeptide chain with multiple transmembrane domains. This discussion will focus upon the thrombin receptor, the most novel of these receptors.

Thrombin receptor structure. When added to platelets *in vitro*, thrombin causes phosphoinositide hydrolysis, eicosanoid formation, an increase in cytosolic Ca^{++} and protein phosphorylation on serine, threonine and tyrosine residues. Thrombin also suppresses cAMP synthesis. All of these responses require thrombin to be proteolytically active and many, if not all, appear to be mediated by G proteins. Until recently, however, the thrombin receptor had not been identified and there was no satisfactory explanation for the mechanism of its activation. Binding studies by Harmon and Jamieson (6) suggested that there were approximately 50 high affinity thrombin binding sites per platelet with a K_d of 0.3 nM, and 1700 moderate affinity sites/platelet with a K_d of 11 nM. Similar results were obtained by De Marco et al. (7). Indirect evidence suggested that the high affinity binding sites might be associated with membrane glycoprotein Ib. However, there was no evidence that an interaction between GP Ib and thrombin evoked a functional response.

In 1991, two laboratories using expression cloning in *Xenopus laevis* oocytes independently reported the successful isolation of similar cDNA clones encoding thrombin receptors from the human megakaryoblastic Dami cell line (3) and from hamster fibroblasts (4). The structure of the Dami cell throm-

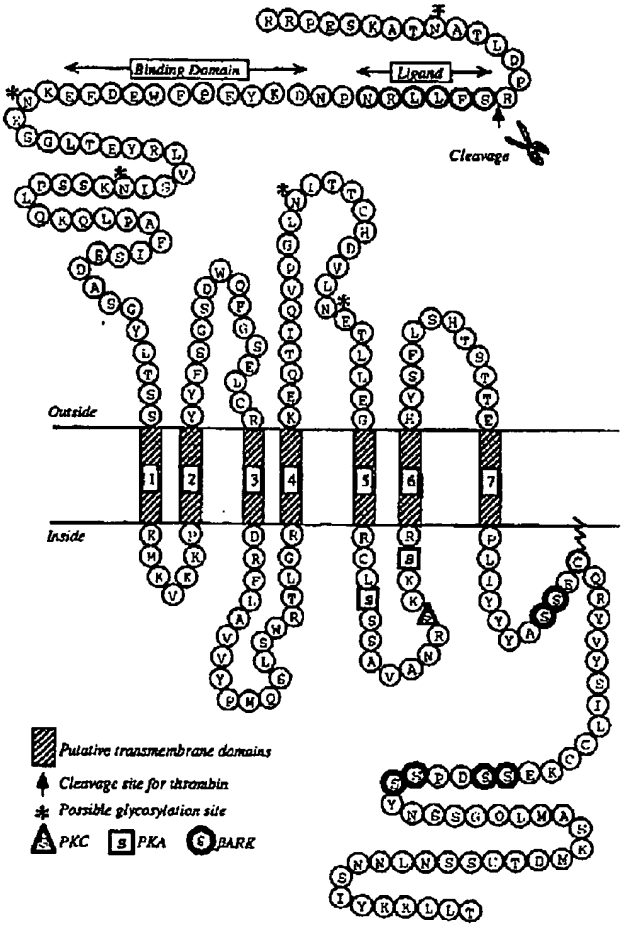

Figure 2: Thrombin receptor structure. Some of the proposed features of the cloned human platelet thrombin receptor (3) are shown, including potential sites for cleavage by thrombin, N-linked glycosylation, and possible phosphorylation by either protein kinase C (PKC), cAMP-dependent protein kinase (PKA) or a kinase analogous to β-adrenergic receptor kinase (βARK). The acidic domain in the N-terminus thought to bind to thrombin's exosite is indicated, as is the SFLLRN sequence thought to serve as the receptor's tethered ligand. For simplicity, the amino acid residues corresponding to the putative transmembrane domains have been omitted from the drawing.

bin receptor, which appears to be identical to the platelet thrombin receptor, is illustrated in Figure 2. Like most other receptors that interact with G proteins, it consists of a single polypeptide chain with seven hydrophobic domains, an extended extracellular N-terminus, and sites for posttranslational processing including palmitoylation of the C-terminus and N-linked glycosylation of the N-terminus. Most notably, a potential site for cleavage by thrombin was found between Arg^{41} and Ser^{42}, and mutations at this site prevented activation of the expressed receptor by thrombin (3,8).

Thrombin receptor activation. Based upon the existence of a thrombin cleavage site within the N-terminus of the receptor, Vu et al. (3) proposed that the N-terminus distal to the point of cleavage forms a tethered ligand capable of activating the receptor. In support of this hypothesis, a 14 residue peptide corresponding to residues Ser^{42} thru Phe^{55} (SFLLRNPNDKYEPF) was shown to cause platelet aggregation (3), and mutations in the tethered ligand domain were found to inhibit activation of the expressed receptor (9). In the two years since publication of this model, many of its key features have been confirmed by several laboratories and ever-growing numbers of observations. Receptor-derived peptides can evoke many, if not all, of the effects of thrombin on platelets, as well as a variety of other cells, including fibroblasts, smooth muscle cells, renal mesangial cells, neurons, monocytes and endothelial cells. The first five or six residues of the tethered ligand domain, SFLLRN, are sufficient to activate the receptor (9-12) and antibodies directed against this domain inhibit platelet activation by thrombin (13,14). Those same antibodies recognize a 65 kDa protein in platelet membranes and bind to approximately 1800 sites per platelet (13), a number in good agreement with the number of moderate affinity thrombin binding sites reported by Harmon and Jamieson (6). The discrepancy between the apparent size of the receptor on SDS gels and the 47 kDa predicted from the amino acid content of the receptor presumably results at least in part from N-linked glycosylation, but this has not been formally proven. Notably, however, translation of the human receptor in a reticulocyte lysate preparation yields a radiolabeled protein close to the predicted size that can be immunoprecipitated by an anti-thrombin receptor antibody (unpublished observation).

Thrombin receptor desensitization. In a variety of thrombin-responsive cells, including platelets, an initial encounter with thrombin produces a state of homologous desensitization in which a subsequent encounter evokes little or no response. In theory, this desensitization could involve any of the mechanisms known to affect other G protein-coupled receptors and, in addition, might arise from a secondary proteolytic event that disables the exposed tethered ligand domain of the receptor. The onset of desensitization is not, however, dependent upon the mechanism by which the receptors are initially activated. Using the megakaryoblastic HEL and CHRF-288 cell lines as models, we found that activation by either thrombin or the tethered ligand domain peptide SFLLRN prevented a subsequent response to both, even though responses to other agonists were preserved (15,16). Notably, recovery from the desensitized state caused by an initial encounter with thrombin occurred in two distinct phases. In the first, which was detectable within 30 minutes, there was a partial recovery of responsiveness to SFLLRN, but no recovery of the response to thrombin. This phase of recovery could be delayed by inhibitors of serine/threonine phosphatases, but was unaffected by cycloheximide. In the second phase of recovery, which required as much as 20 hrs to complete, there was a full return of receptor responses to both SFLLRN and thrombin. This phase of recovery could be inhibited by cycloheximide, suggesting that it was at least partly due to the synthesis of new receptors (16).

These results suggest that phosphorylation and dephosphorylation play a role in the regulation of thrombin receptor function. They also suggest that the synthesis of new receptors is required for a full recovery of the response to thrombin in cells that are capable of synthesizing receptors. Recent studies with receptor-directed monoclonal antibodies have

helped to extend this model; within two minutes of the addition of thrombin, >90% of the thrombin receptors on HEL and CHRF-288 cells are internalized, appearing first in coated vesicles within the plasma membrane and then in endosomes (17). This process occurs equally well in response to SFLLRN or thrombin, and therefore does not require cleavage of the receptor's N-terminus. Whether the receptors are desensitized before they are internalized remains to be determined. From the endosomes, approximately three-quarters of the receptors are transferred to lysosomes and degraded. The remainder are recycled back to the cell surface in a state in which they can respond to SFLLLRN, but not to thrombin; this accounts for the observation that thrombin-desensitized cells recover their ability to respond to SFLLRN sooner than they recover their ability to respond to thrombin. The apparent failure of the recycled receptors to be auto-activated by their tethered ligand domain may have implications for the role of thrombin in the presumed interaction of this portion of the N-terminus with the remainder of the receptor.

If phosphorylation proves to play a role in thrombin receptor desensitization, what can be said about the protein kinase(s) that may be involved and about potential sites for phosphorylation? Present evidence suggests that at least two different kinases may play a role. The first is protein kinase C, which phosphorylates and desensitizes other G protein-coupled receptors. Direct activators of protein kinase C such as phorbol esters inhibit signaling through platelet thrombin receptors (18,19). Thrombin receptors contain at least one consensus sequence for phosphorylation by protein kinase C, a serine residue within the third cytoplasmic loop (Fig. 2). Could activation of protein kinase C account for thrombin receptor desensitization and internalization? Several observations suggest that this is not the case. First, while the desensitization caused by thrombin affects only the response to thrombin, the desensitization caused by phorbol esters (TPA) affects responses to other agonists as well. Second, the protein kinase C inhibitor staurosporine completely blocks the desensitization caused by TPA, but has only a minimal effect on the desensitization caused by thrombin (16). Finally, although our preliminary observations show that TPA stimulates receptor internalization, the kinetics are considerably slower than internalization caused by thrombin or the loss of function caused by TPA.

An alternative to protein kinase C is a receptor-specific protein kinase such as the β-adrenergic receptor kinase (βARK) or rhodopsin kinase. At present there is no direct evidence that a kinase with specificity for the thrombin receptor exists. However, βARK can phosphorylate both α2-adrenergic and β-adrenergic receptors, and both HEL cells and platelets contain an ≈80 kDa protein recognized by rabbit polyclonal anti-βARK antiserum (unpublished observation). It is conceivable that it or a related kinase could phosphorylate thrombin receptors. The sites for phosphorylation by βARK in β- and α2-adrenergic receptors are located in the third cytoplasmic loop and in the C-terminus. Studies with synthetic substrates suggest that βARK prefers serine residues to threonine residues and that an acidic residue on the amino side of the serine residue enhances phosphorylation (20). As is indicated in Figure 2, there are at least four such serine residues in the C-terminus of the thrombin receptor. Again, however, the evidence that activated thrombin receptors become phosphorylated is still indirect and there is no published evidence for an interaction between thrombin receptors and βARK.

Unresolved issues. Far from answering all of the questions about the mechanisms of platelet activation by thrombin and other agonists, these observations leave a number of issues unresolved. The first is whether platelets have thrombin receptors in addition to the one that has been cloned. Although such a possibility has not been ruled out, the failure of Northern and Southern blots to give evidence of a second receptor, and the ability of peptides such as SFLLRN to mimic many, if not all, of the effects of thrombin on platelets suggests that only a single class of receptors may be present. A second issue is whether the "thrombin" receptor is really a more generalized protease receptor and, if so, what implications this has for platelet activation at sites of inflammation and wound repair. Platelets can be activated by trypsin, cathepsin G and plasmin, as well as thrombin. In theory, any protease capable of cleaving the receptor N-terminus would activate the receptor, although the site of cleavage would presumably have to be between Arg^{41} and Ser^{42}. Trypsin mimics many of the effects of thrombin on platelets and HEL cells (16), and the anti-receptor antibodies that inhibit platelet activation by thrombin also inhibit platelet activation by trypsin (13). Further work using these approaches should help to determine whether the thrombin receptor responds to other proteases and whether exposure of an occult tethered ligand is involved in the response of other receptors to other types of proteases. A third issue is the role of protein tyrosine kinases in platelet responses to thrombin. Such kinases become activated during platelet activation, although apparently in a largely aggregation-dependent manner (21). Whether direct interactions between G proteins, G protein coupled receptors and tyrosine kinases occur early in platelet activation remains to be determined. A final issue is the identity of platelet receptors for other key agonists, particularly collagen and ADP. Platelet binding to collagen is thought to be mediated by the GP Ia-IIa complex and GP Ib. Although the evidence for collagen interactions with all of these glycoproteins is compelling, none of them have been shown to be a receptor in the functional sense. Similarly, ADP binding proteins have been identified on platelets, but not yet shown to be functional receptors.

G proteins

Guanine nucleotide-binding regulatory proteins or G proteins mediate the interaction between cell surface receptors, such as those for thrombin, and intracellular or plasma membrane effectors such as phospholipases and ion channels. G proteins have a characteristic heterotrimeric structure in which the α subunit contains the guanine nucleotide binding site and, in some cases, is a substrate for ADP-ribosylation by bacterial toxins. ADP-ribosylation by cholera toxin or pertussis toxin alters G protein function, either by mimicking the effects of

Table 1: G Protein α subunits in platelets

	kDa	Toxin Sensitivity	Phosphorylated	Affected Enzyme	Function	Ref.
$G_{i\alpha2}$>>$G_{i\alpha3}$>$G_{i\alpha1}$	40-41	pertussis	no	adenylyl cyclase, phospholipase C ?	↓ cAMP, ↑ IP_3/DAG ?	(32)
$G_{z\alpha}$	41	neither	yes	?	?	(47,48,52)
$G_{q\alpha}$, $G_{11\alpha}$	42	neither	no	phospholipase C	↑ IP_3/DAG	(40) and unpublished observations
$G_{12\alpha}$, $G_{13\alpha}$	44	neither	?	?	?	unpublished observations
$G_{s\alpha}$	45	cholera	no	adenylyl cyclase	↑ cAMP	(53)

an agonist-occupied receptor or by inhibiting agonist-evoked responses. An example of the former is the effect of cholera toxin on G_s, the G protein which stimulates cAMP formation. ADP-ribosylation of $G_{s\alpha}$ causes continuous activation of adenylyl cyclase. In contrast, ADP-ribosylation by pertussis toxin can inhibit responses to agonists. An example of this effect in platelets is the ability of pertussis toxin to block the G_i-mediated suppression of cAMP formation caused by agonists such as thrombin and epinephrine. G protein β and γ subunits perform at least two roles. First, they form an isoprenylated heterodimer ($G_{\beta\gamma}$) which helps to anchor the G protein to cell membranes. Second, they have regulatory effects on adenylyl cyclase and phospholipases that are independent of the α subunit (22-28). G proteins are traditionally thought to regulate events at the plasma membrane, but recent studies reveal that G proteins can also associate with cytoplasmic structures (29,30), an association whose role remains to be determined.

Historically, G_s and G_i were among the first G proteins described in both structural and functional terms. G_s and G_i regulate cellular cAMP levels by, respectively, stimulating and inhibiting adenylyl cyclase. Recent cloning studies have shown that most forms of G_α, including $G_{s\alpha}$ and $G_{i\alpha}$, exist as families of several closely-related proteins (31). There are, for example, at least three different forms of $G_{i\alpha}$: $G_{i\alpha1}$, $G_{i\alpha2}$ and $G_{i\alpha3}$. These three proteins are 85-95% homologous with each other at the amino acid level. All three are substrates for pertussis toxin. The significance of the differences between the members of the $G_{i\alpha}$ family is unknown. However, the preservation of the differences across tissue and species lines suggests that the proteins may play distinct roles. In addition to adenylyl cyclase, G proteins have been implicated in the regulation of K^+ and Ca^{++} channels, hydrolysis of phosphatidylcholine, and the activation of phospholipases A_2 and C and cGMP phosphodiesterase. At the time that this review was written, at least 22 forms of G_α, four forms of G_β and seven forms of G_γ had been described, yielding hundreds of possible combinations, not all of which appear to exist in nature. The factors determining which combinations occur and the relative contributions of G_α and $G_{\beta\gamma}$ to the regulation of effector function are topics of considerable current interest.

G proteins in platelets. In platelets, as in other cells, G proteins play a major role in signal transduction by mediating the interaction between agonist receptors and the enzymes which produce second messengers (Fig. 1). The nine forms of G_α that have been identified in platelets are shown in Table 1. Based upon their functional characteristics, Western blotting with peptide-directed antibodies, ADP-ribosylation by bacterial toxins, Northern blots and nucleotide sequencing, platelets contain both G_s and G_i. $G_{s\alpha}$ appears to be present in at least its 45 kDa form. $G_{i\alpha}$ is present in all three of its known forms, but not with equal abundance (32). G_s and G_i are thought to play the same role in the regulation of cAMP formation in platelets that they do in other cells. Agents which increase cAMP levels in platelets, such as prostaglandin I_2, do so via G_s. Agonists such as thrombin and epinephrine, which suppress cAMP formation, are thought to do so through G_i.

In addition to adenylyl cyclase, G proteins appear to regulate at least two other platelet enzymes: phospholipase C and phospholipase A_2. Regulation of phospholipase C by a G protein is a general phenomenon that occurs in a wide variety of cells and presumably involves the β forms of phospholipase C. The G protein that stimulates phospholipase C is sometimes referred to generically as "G_p". However, recent studies suggest that G_p may not be the same in all cells, or even a single G protein. In some cells, particularly those of hematopoietic origin, agonist or hormone-induced phosphoinositide hydrolysis can be blocked by preincubating the cells with pertussis toxin. In other cells, pertussis toxin has no effect. Studies in platelets have been complicated by the inability of pertussis toxin to cross the platelet plasma membrane. However, pertussis toxin can inhibit thrombin-induced phosphoinositide hydrolysis when introduced into permeabilized platelets (33) or when added to intact HEL cells (15). These observations suggest that platelets contain at least one form of G_p that is a substrate for pertussis toxin. Since all of the pertussis toxin substrates in platelets that have been identified to date are members of $G_{i\alpha}$ family, G_{i1}, G_{i2} and/or G_{i3} may regulate phospholipase C as well as adenylyl cyclase. This has not, however, been proven. Reconstitution experiments in which activated $G_{i\alpha}$ was combined with phospholipase C have failed to show a substantial increase in phospholipase C activity. However, recent studies showing that $G_{\beta\gamma}$ can stimulate the β2 form of phospholipase C may provide a

solution: if thrombin receptors interact primarily with Gi in platelets, then the availability of G_i-derived $G_{\beta\gamma}$ in thrombin-treated platelets would be reduced in the presence of pertussis toxin. However, this explanation leaves unexplained the failure of epinephrine to stimulate phospholipase C, as well as to inhibit adenylyl cyclase.

Platelets are also thought to contain a pertussis toxin-resistant G protein capable of stimulating phospholipase C. The initial evidence for this conclusion was indirect: under conditions in which thrombin-induced phosphoinositide hydrolysis was inhibited by pertussis toxin, the ability of the TxA_2 analog U46619 to activate phospholipase C was unimpaired (34,35). Subsequently, several new forms of G_α were described that could activate phospholipase C (36-39). Of these, $G_{q\alpha}$ and $G_{11\alpha}$ are present in platelets ((40) and unpublished observations). Neither possesses the site at which ADP-ribosylation by pertussis toxin normally occurs and neither is thought to be a substrate for the toxin. Reconstitution studies with the β1 subtype of phospholipase C and purified or recombinant $G_{q\alpha}/G_{11\alpha}$ show that these proteins can cause a five-fold or greater increase in phosphoinositide hydrolysis by the enzyme (41-44). It is assumed, therefore, that one or both of these proteins mediates pertussis toxin-resistant activation of phospholipase C in most cells. Although this has not been specifically established in platelets, Shenker et al. (40) have shown that an antibody directed against $G_{q\alpha}/G_{11\alpha}$ can inhibit thromboxane receptor-stimulated GTPase activity. Therefore, it appears likely that the various β forms of phospholipase C in platelets are regulated by several different mechanisms involving one or more forms G_α and $G_{\beta\gamma}$. An entirely separate mechanism involving tyrosine phosphorylation is thought to regulate the activity of phospholipase C_γ. A recent review discussing the regulation of phospholipase C is found in reference 45.

Phospholipase A_2 is the second phospholipid-hydrolyzing enzyme in platelets whose activity may be regulated by G protein(s). Two potential mechanisms could be involved. One is the indirect activation of phospholipase A_2 by the increase in cytosolic Ca^{++} caused by G_i- or G_q-dependent phosphoinositide hydrolysis. The second is the direct activation of phospholipase A_2, which has been shown in other types of cells to be mediated by $G_{\beta\gamma}$ (46). Current evidence suggests that phospholipase A_2 is primarily located in the platelet cytosol and that arachidonate release and metabolism occur in the dense tubular system. However, both mechanisms may be active in platelets, perhaps with different forms of phospholipase A_2 that are regulated in different ways. This is a long-unresolved issue that remains to be adequately addressed.

In addition to the G proteins discussed thus far, platelets contain several newly-described G proteins. One is $G_{z\alpha}$, which is identical to a protein whose DNA has been cloned from brain and retinal cDNA libraries (47,48). Like $G_{q\alpha}$ and $G_{11\alpha}$, $G_{z\alpha}$ is not a substrate for pertussis toxin. Like other forms of G_α, $G_{z\alpha}$ is able to interact with $G_{\beta\gamma}$ and hydrolyze GTP to GDP, although at a rate slower than other G proteins. G_z has one interesting property, however, that is so far unique among platelet G proteins and is of considerable interest. When platelets are activated by thrombin or TxA_2 analogs, both of which activate protein kinase C via phosphoinositide hydrolysis, or by phorbol esters, which directly activate protein kinase C, $G_{z\alpha}$ is phosphorylated (47). This phosphorylation occurs with a stoichiometry of one mole of phosphate incorporated per mole of $G_{z\alpha}$, can be reproduced with recombinant $G_{z\alpha}$ and purified protein kinase C, and takes place at Ser^{27} (49,50). Under the same conditions, the forms of $G_{i\alpha}$ present in platelets are not phosphorylated (47). The biological role of G_z and of $G_{z\alpha}$ phosphorylation are still unknown.

In addition to $G_{z\alpha}$, transcripts encoding three additional forms of G_α have recently been described, $G_{12\alpha}$, $G_{13\alpha}$ and $G_{16\alpha}$, all of which are now included in the $G_{q\alpha}$ "family" (24,31,51). Based upon their amino acid sequences, none of these are predicted to be pertussis toxin substrates. By RNA analysis after PCR amplification, $G_{12\alpha}$ and $G_{13\alpha}$ are widely distributed (31), while $G_{16\alpha}$ is found predominantly in hematopoietic cells (51). Peptide-directed antisera detect $G_{12\alpha}$ and $G_{13\alpha}$ in platelets, but not $G_{16\alpha}$ (unpublished observation).

Unresolved issues. Although the list of the G proteins present in platelets has grown steadily over the past several years, many questions remain unanswered (Table 1). For example, what are the mechanisms by which G proteins regulate the activity of phospholipase C and phospholipase A_2, particularly in situations where pertussis toxin prevents the activation of these enzymes? What are the relative contributions of G_α and $G_{\beta\gamma}$ to the regulation of these enzymes? What is the role of G_z in platelets and the effects of phosphorylation on that role? The tissue distribution of $G_{z\alpha}$ is limited; it is present in megakaryocytes, but absent from the non-megakaryocytic hematopoietic cells that we have examined and from the megakaryoblastic HEL, Dami and CHRF-288 cell lines ((48) and unpublished observations). G_z may plays a role during megakaryocyte development, but this remains to be established. A final issue is the role of G proteins located at sites other than the plasma membrane; although intracellular locations have not yet been demonstrated in platelets, data from other types of cells make it likely that they exist in platelets.

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The human platelet ADP receptor activates G_{i2} proteins

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We have previously shown that platelet ADP receptors are coupled to G-proteins by measuring the binding of [^{35}S]guanosine-5'-[γ-thio]triphosphate ([^{35}S]GTPγS) to human platelet membranes stimulated with ADP. In order to identify the activated G-proteins, we used an approach which combines photolabelling of receptor-activated G-proteins with 4-azidoanilido-[α-^{32}P]GTP and immunoprecipitation of the G-protein α-subunits with subtype-specific antibodies. Stimulation of human platelet membranes with ADP resulted in an increase in 4-azidoanilido-[α-^{32}P]GTP incorporation into the immunoprecipitates of $G\alpha_i$ but not of $G\alpha_q$ proteins, whereas stimulation with the thromboxane analogue U46619 resulted in an increase in 4-azidoanilido-[α-^{32}P]GTP incorporation into the immunoprecipitates of $G\alpha_q$ but not of $G\alpha_i$ proteins, and thrombin activated both G-proteins. This effect of ADP was concentration dependent and inhibited by the class P_2 purinoceptor (P_{2T}) antagonist ATP. Using specific antisera against subtypes of G_i proteins, we found that ADP stimulated labelling of the $G\alpha_{i2}$ immunoprecipitate, but not of the $G\alpha_{i3}$ precipitate. $G\alpha_{i1}$ was not detectable by immunoblotting of platelet membrane proteins. These data suggest that ADP inhibits cAMP formation by activation of $G\alpha_{i2}$ proteins and add evidence in support of the hypothesis that human platelet ADP receptors do not activate PLC through G_q activation.

INTRODUCTION

Platelet aggregation by ADP plays a key role in the development and extension of arterial thrombosis. Stored at very high concentrations in platelet dense granules, ADP is released when platelets are stimulated by other aggregating agents, such as thrombin or collagen, and so contributes to and reinforces platelet aggregation. In addition, ADP at low concentrations potentiates or amplifies the effects of all other stimuli, even weak agonists such as adrenaline or serotonin [1–3]. Addition of ADP to washed human platelet suspensions results in shape change, exposure of the fibrinogen-binding site on the $\alpha_{IIb}\beta_3$ integrin and reversible aggregation in the presence of fibrinogen and physiological concentrations of Ca^{2+}. At the intracellular level, platelet activation following ADP binding to its receptor leads to a transient rise in free cytoplasmic Ca^{2+}, resulting from both Ca^{2+} influx and mobilization of internal stores. ADP also inhibits stimulated adenylyl cyclase [1], but this is not causally involved in platelet aggregation.

On the basis of agonist selectivity and signalling properties [4,5], the platelet receptor for ADP has been classified as a P_{2T} receptor of the P_2 purinoceptor family. Its main characteristic is that ADP is its natural agonist while ATP is a competitive antagonist. However the biochemical structure of this receptor remains unknown. Most of the platelet membrane receptors such as platelet-activating factor, thromboxane A_2 (TXA_2), thrombin, adrenaline and 5'-hydroxytryptamine belong to the seven transmembrane domain G-protein-coupled receptor family, and many have been cloned and sequenced [6]. Several ADP-binding proteins have been proposed as putative ADP receptors, for instance the 100 kDa protein called aggregin [7] or more recently a 40 kDa protein which incorporates a photoaffinity analogue of ADP [8]. Nevertheless, the P_{2T} receptor has not yet been precisely identified by biochemical or molecular biological techniques. Three receptor subtypes of the P_2 family have been cloned, sequenced and expressed: G-protein-coupled receptors in the form of mouse and human P_{2U} receptors and a chicken P_{2Y} receptor, and recently, channel-linked P_{2X} receptors from rat vas deferens and pheochrocytoma PC12 cells [9]. Whether the P_{2T} receptor is a G-protein-coupled or a channel-linked receptor is not known. It has been proposed that there could exist more than one type of ADP receptor in blood platelets, since the ADP-binding protein seems to possess characteristics of both known types [3]. In contrast to the other P_2 purinoceptors, which are broadly expressed in many tissues, the P_{2T} receptor(s) appears to be specific for platelets and has never been found in other cells, apart from certain studies where functional properties suggested that this receptor could be expressed by K562 [10] and Dami leukaemia cells [11], or where functional and binding characteristics indicated its presence in the megakaryoblastic cell line Meg-01 [12]. Obviously, a better knowledge of the ADP platelet-activation pathway is of major importance for the identification of this receptor and the understanding of the physiology of primary haemostasis.

We have previously shown that ADP stimulates the binding of [^{35}S]guanosine-5'-[α-thio]triphosphate ([^{35}S]GTPγS) to platelet membranes in humans and in rats [13,14], suggesting that the ADP receptor is coupled to G-proteins, the nature of which remain to be identified. The aim of the present study was the identification of these activated G-proteins. For this purpose, we used an approach which combines photolabelling of receptor-activated G-proteins with 4-azidoanilido-[α-^{32}P]GTP and immunoprecipitation of the G-protein α-subunits with subtype-specific antibodies [15].

MATERIALS AND METHODS

Chemicals

[α-^{32}P]GTP was obtained from Du Pont-New England Nuclear

Abbreviations used: GTP[γ]S, guanosine-5'-O-(3-thiotriphosphate); $G\alpha_i$, α-subunit of the G_i class of G-proteins; P_{2T}, platelet purinoceptor of class P_2; cAMP, cyclic AMP; PLC, phospholipase C; IP_3, D-*myo*-inositol 1,4,5-triphosphate; TXA_2, thromboxane A_2; ECL, enhanced chemiluminescence; KLH, keyhole limpet haemocyanin; *N*-DEC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl; NP 40, Nonidet P-40.

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(Boston, MA, U.S.A.) and 4-azidoaniline HCl and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (*N*-DEC) from Fluka (Buchs, Switzerland). All nucleotides were from Boehringer Mannheim (Mannheim, Germany). U46619 (TXA_2 receptor agonist) was from Sigma (St. Louis, MO, U.S.A.), while the thrombin receptor-activating peptide (TRAP-SF7) was synthesized by Neosystem (Strasbourg, France).

Antibodies

Rabbit polyclonal antibodies were raised against peptides corresponding to specific regions of G-protein α-subunits and coupled to keyhole limpet haemocyanin (KLH) with an additional cysteine at the N-terminus as previously described [16]. The antisera were designated as follows: AS 8 (anti-α common); AS 190 (anti-α_{i1}); AS 269 (anti-α_{i2}); AS 105 (anti-α_{i3}); AS 266 (anti-α_{i3}); AS 266 (anti-α_i common); AS 227 (anti-α_z); AS 369 (anti-$\alpha_{q/11}$); AS 232 (anti-α_{12}); and AS 343 (anti-α_{13}). All were of known characteristics [15,17,18] and were employed as crude sera for immunoprecipitation and immunoblotting.

Platelet membrane preparation

Human platelets were isolated and washed as previously described [19], and resuspended in Tyrode's buffer, without Ca^{2+}, containing 2 mM EDTA, apyrase (2 μg/ml) and protease inhibitors [leupeptin (10 μM), aprotinin (1 μM), PMSF (20 μM)]. The platelet suspension was frozen and thawed before being homogenized in a glass–Teflon homogenizer on ice. Membranes were pelleted by centrifugation (30000 *g* for 15 min) at +4 °C and the final pellet was resuspended in 50 mM Tris/HCl (pH 7.5) containing 2 mM EDTA and stored at − 80 °C. Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay system (Pierce, Rockford, IL, USA).

Synthesis and purification of [α-^{32}P]GTP-azidoanilide

[α-^{32}P]GTP-azidoanilide was synthesized using a modification of a standard method [20]. Briefly, [α-^{32}P]GTP (5 mCi/111 TBq/mmol) was evaporated to dryness under vacuum and dissolved in 120 μl of 100 mM Mes buffer (pH 5.5) containing 1.2 mg of *N*-DEC. After 15 min incubation under rotation at room temperature in the dark, 1.3 mg of azidoaniline was added and the reaction was allowed to proceed for 8 h. Under these conditions, the synthesis yield was 70 %. Purification was performed by HPLC [20], [α-^{32}P]GTP-azidoanilide was adjusted to 74 kBq/μl and aliquots were stored at −80 °C.

Photolabelling of membrane proteins

Platelet membranes (400 μg of protein per sample) were suspended in ice-cold incubation buffer containing 50 mM Hepes (pH 7.4), 0.1 mM EDTA, 10 mM $MgCl_2$, 30 mM NaCl, 1 mM benzamidine and 0.2 % (w/v) BSA. In some cases, GDP (1–10 μM) was added to the incubation buffer [15]. After a 10 min preincubation in the absence or presence of agonists, the samples were incubated for a further 10–20 min, depending on the G_α-subunit, with [α-^{32}P]GTP-azidoanilide (5 μCi/tube). The reaction was stopped by cooling on ice and all subsequent procedures were performed at +4 °C. Samples were centrifuged (12000 *g* for 5 min), the membrane pellets were resuspended in the same buffer supplemented with 2 mM glutathione and these samples were then irradiated for 30 s at +4 °C with a UV lamp (254 nm, 100 W) at a distance of 3 cm. After irradiation, the samples were centrifuged again (12000 *g* for 5 min) and solubilized in SDS buffer as described below.

Immunoprecipitation

Immunoprecipitation was performed by standard techniques [15,17]. Pellets of photolabelled membranes were solubilized in 40 μl of 2 % (w/v) SDS at room temperature. Following addition of 120 μl of precipitating buffer containing 1 % (v/v) Nonidet NP.40 (NP40) or 1 % (w/v) Chaps, 1 % (w/v) deoxycholate, 0.5 % (w/v) SDS, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 20 μM PMSF, 1 μM aprotinin and 10 mM Tris/HCl (pH 7.4), samples were incubated with 15–20 μl of antiserum for at least 90 min at +4 °C under constant rotation. Washed Protein A-Sepharose beads [60 μl of a 10 % (w/v) suspension in precipitating buffer] were added to the supernatants and incubated overnight at +4 °C, still under rotation. The Sepharose beads were then pelleted (12000 *g* for 5 min) and washed, first with 2 × 1 ml of high-salt buffer containing 1 % (v/v) NP40, 0.5 % (w/v) SDS, 600 mM NaCl and 50 mM Tris/HCl (pH 7.4), and then with 1 ml of low-salt buffer containing 300 mM NaCl, 10 mM EDTA and 100 mM Tris/HCl (pH 7.4). Finally, the samples were solubilized in Laemmli buffer and subjected to SDS/PAGE [21]. Electrophoresis was performed on 10 % (w/v) acrylamide gels and run until the 30 kDa standard protein reached the bottom of the gel. The photolabelled proteins were visualized by autoradiography of the dried gels, densitometric analyses being carried out by means of an image analyser (Biocom, Paris, France).

Immunoblotting

Platelet membrane proteins were separated on 9 % polyacrylamide gels containing 6 M urea for the $G\alpha_i$ subunits and on 13 % polyacrylamide gels without urea for members of the $G\alpha_{q/11}$ and $G\alpha_{12}$ families. Immunodetection of $G\alpha$ subunits was performed by the enhanced chemiluminescence (ECL) procedure (Amersham, Little Chalfont, U.K.) as described in [17].

Reproducibility

The experiments shown are representative of at least three independent assays using different membrane preparations.

RESULTS

Immunoblotting

Immunoblotting of platelet membranes with antisera raised against peptides corresponding to specific regions of G-protein α-subunits (see Table 1) confirmed that platelets express G_i-.

Table 1 Peptide antisera used for the detection of G-protein α-subunits

(C) indicates that an N-terminal cysteine was added to the original peptide sequence in order to facilitate the coupling to KLH

Antiserum	Peptide sequence	G-protein α-subunit recognized
AS 8	(C) GAGESGKSTIVKQM	$\alpha_{i1}/\alpha_{i2}/\alpha_{i3}$
AS 266	(C) NLREDGEKAAREV	$\alpha_{i1}/\alpha_{i2}/\alpha_{i3}$
AS 190	(C) IDRIAQPNYI	α_{i1}
AS 269	(C) TGANKYDEAAS	α_{i2}
AS 105	(C) LDRISQSNYI	α_{i3}
AS 369	(C) LQINLKEYNLV	α_{q11}
AS 232	(C) QENLKDIMLQ	α_{12}
AS 343	(C) LHDNLKQLMLQ	α_{13}
AS 227	(C) HLRSESQHQRREI	α_z

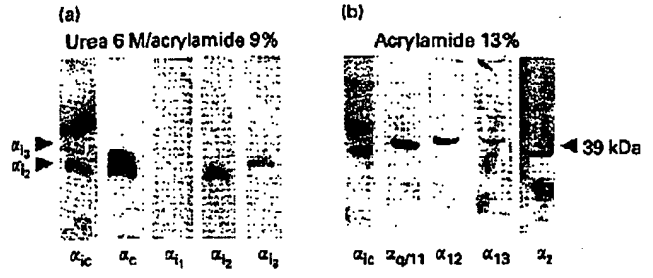

Figure 1 Immunoblot analysis G-protein α-subunits in platelet membranes

Platelet membrane proteins (200 μg) were separated on SDS/9% acrylamide gels containing 6 M urea for the $G\alpha_i$ subtype (a) and on SDS/13% acrylamide gels for the other subtypes (b) and blotted onto nitrocellulose filters. Filter strips were incubated with the following antisera (specificity, dilution): AS 266 (anti-α_{ic}, 1:25); AS 8 (anti-α_c, 1:50); AS 190 (anti)α_{11}, 1:25); AS 269 (anti-α_{i2}, 1:50); AS 105 (anti-α_{i3}, 1:50); AS 369 (anti-$\alpha_{q/11}$, 1:150); AS 232 (anti-α_{12}, 1:50); AS 343 (anti-α_{13}, 1:50); and AS 227 (anti-α_z, 1:100). Bound antibodies were visualized by the ECO technique as described in the Materials and methods section.

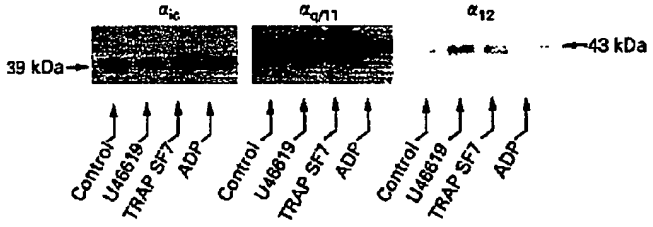

Figure 2 Photolabelling and immunoprecipitation of $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$

Platelet membranes (400 μg) were photolabelled with 5 mCi of [α-^{32}P]GTP-azidoanilide, in the absence of a stimulator (control) or in the presence of 5 μM U46619, 10 μM TRAP-SF7 or 10 μM ADP. Proteins were immunoprecipitated with antisera AS 266 (anti-α_c, 20 μl), AS 369 (anti-$\alpha_{q/11}$, 15 μl) and AS 232 (anti-α_{12}, 15 μl), separated on SDS/10% acrylamide gels and revealed by autoradiography.

G_q- and G_{12}-proteins [17], with the exception of $G\alpha_{11}$ which was not recognized by our antibody (Figure 1). This absence of $G\alpha_{11}$ was supported by the observation that purification of pertussis toxin-sensitive G-proteins from human platelets yielded only $G\alpha_{12}$ and $G\alpha_{13}$ (results not shown). Using a 6 M urea/9% acrylamide separating gel, we were able to distinguish between $G\alpha_{12}$ and $G\alpha_{13}$ with an anti-$G\alpha_i$ common antibody (AS 266) and this result was confirmed with the subtype-specific antibodies raised against $G\alpha_{i2}$ (AS 269) and $G\alpha_{i3}$ (AS 105). Once again, $G\alpha_{11}$ was not detectable. In addition, the antibody (AS 369) specific for the G-protein α-subunits α_q and α_{11} showed the presence of a 42 kDa protein corresponding to α_q, as reported earlier for α_{12}, α_{13} and α_z [22].

Photolabelling of membrane proteins and immunoprecipitation

In order to identify the G-protein(s) activated by ADP, platelet membranes were photolabelled with [α-^{32}P]GTP-azidoanilide in the absence and presence of 10 μM ADP and immunoprecipitation was performed using different subtype-specific antibodies. As controls, platelet membranes were also stimulated with the TXA_2 receptor agonist U46619 (5 μM) and the thrombin receptor agonist TRAP-SF7 (10 μM). As expected from previous studies [17], U46619 stimulated the photolabelling of $G\alpha_q$, $G\alpha_{i3}$ (Figure 2) and $G\alpha_{12}$ proteins (results not shown) without any effect on $G\alpha_i$, where TRAP-SF7 stimulated $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$

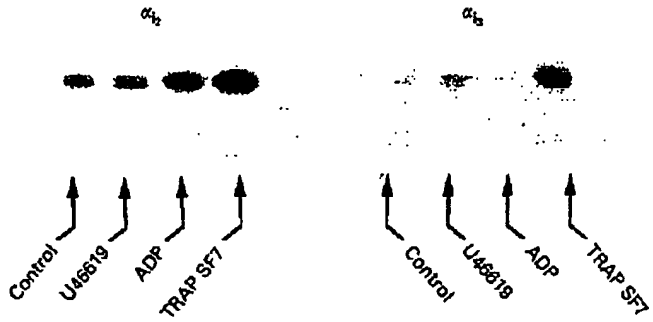

Figure 3 Photolabelling of $G\alpha_i$ subtypes under ADP and thrombin receptor stimulation

Membranes (400 μg) were incubated in a medium containing 10 μM GDP and photolabelled as described in Figure 2 in the absence of a stimulator (control) or in the presence of 10 μM ADP, 10 μM TRAP-SF7 or 5 μM U46619 (negative control). The samples were then solubilized in a medium containing 1% (w/v) Chaps instead of NP40. Proteins were immunoprecipitated with antisera AS 269 (anti-α_{i2}, 20 μl) and AS 105 (anti-α_{i3}, 20 μl), separated on SDS/10% acrylamide gels and revealed by autoradiography of the dried gels.

(Figure 2) or $G\alpha_{13}$ photolabelling (results not shown). Platelet membrane stimulation with ADP resulted in an increase in [α-^{32}P]GTP-azidoanilide incorporation in the immunoprecipitates of $G\alpha_i$ but not of $G\alpha_q$ proteins (Figure 2). Using $G\alpha_{i2}$ and $G\alpha_{i3}$ subtype-specific antisera, we observed that ADP stimulated the photolabelling of $G\alpha_{i2}$ but not of $G\alpha_{i3}$, while TRAP-SF7 stimulated the labelling of both $G\alpha_{i2}$ and $G\alpha_{i3}$, (Figure 3). In $G\alpha_i$ subtype-specific immunoprecipitation experiments, the platelet membranes were solubilized with Chaps instead of NP40, which improved the immunoprecipitation of $G\alpha_{i2}$ and $G\alpha_{i3}$ with our antibodies. The stimulatory effect of ADP was concentration dependent from 0.1–10 μM and was inhibited by simultaneous incubation with the P_{2T} receptor antagonist ATP (10 μM). This is illustrated in Figure 4, where Figure 4(a) shows an autoradiogram of a dried gel and Figure 4(b) the corresponding densitogram obtained by image analysis.

DISCUSSION

We have previously shown that ADP stimulates the binding of [^{35}S]GTP[γS] to platelet membranes from humans and rats [13,14], suggesting that the ADP receptor is coupled to G-proteins, the nature of which remain unknown. In order to identify these proteins, we combined photolabelling of receptor-activated G-proteins with immunoprecipitation of the labelled G-protein α-subunits. This method allows exact identification of the receptor-activated G-proteins, provided the necessary subtype-specific antisera are available [15]. All the antibodies used recognized the corresponding G-protein α-subunit in platelets except the anti-$G\alpha_{i1}$ AS 190 antibody. This antibody recognizes $G\alpha_{i1}$ in the human neuroblastoma cell line SH-SY5Y [18] and purified $G\alpha_{i1}$ from bovine brain [23]. The reason why it did not detect any platelet protein is unclear, since small amounts of $G\alpha_{i1}$ have been shown to be present in platelets [24], even though the functional role of $G\alpha_{i1}$ in these cells is not known. However, purification of pertussis toxin sensitive G-proteins from 7 g of platelet membranes yielded $G\alpha_{i2}$ and $G\alpha_{i3}$ but no detectable $G\alpha_{i1}$ (results not shown).

Using the antibodies described, we found that stimulation of the platelet ADP receptor led to an increase in the incorporation of [α-^{32}P]GTP-azidoanilide into the $G\alpha_{i2}$ subunit, while, as expected from previous reports, U46619 activated $G\alpha_q$ and

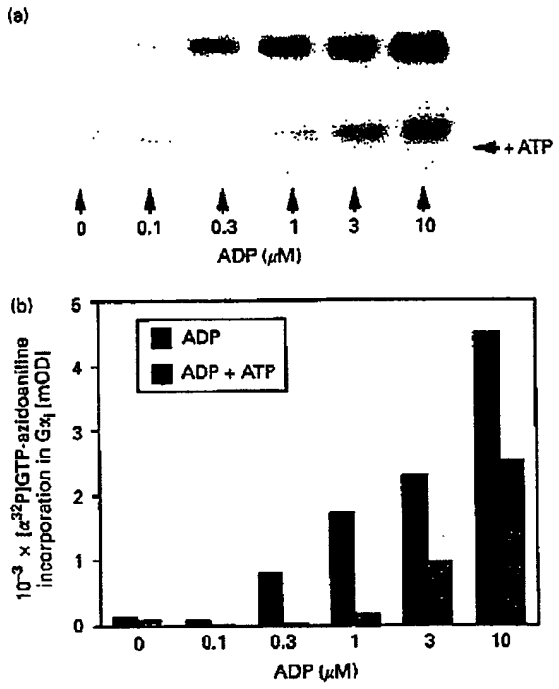

Figure 4 Concentration dependence of the ADP-stimulated photolabelling of $G\alpha_i$

Platelet membranes (400 μg) were incubated in a medium containing 10 μM GDP and photolabelled as described for Figure 2 in the presence of increasing concentrations of ADP (0.1–10 μM), in the absence or presence of 10 μM ATP. Proteins were immunoprecipitated with antiserum AS 266 (anti-$\alpha_{i_{3}}$, 20 μl) and separated on SDS/10% acrylamide gels. The photolabelled proteins were then visualized by autoradiography (**a**) and their relative abundance determined by densitometric image analysis (**b**), results shown here being representative of three identical independent experiments.

thrombin activated both $G\alpha_q$ and $G\alpha_i$ [17]. U46619 activates phospholipase C (PLC) in a pertussis toxin independent manner through G_q. Thrombin or the thrombin receptor agonist peptide (TRAP-SF7) simultaneously inhibits adenylyl cyclase through G_i proteins and activates phospholipase C (PLC) through G_q and presumably by means of $\beta\gamma$ dimers of pertussis toxin sensitive G-proteins [6]. Whereas ADP is known to inhibit platelet adenylyl cyclase, it is a matter of debate whether or not this agonist causes activation of PLC [3]. Our results support the idea that ADP does not activate PLC through the G_q pathway. On the other hand, adrenaline, which is not itself an aggregating agent [25], has been shown to inhibit adenylyl cyclase in human platelets through α_2 adrenergic receptors which are coupled to $G\alpha_{i2}$ [26], without causing any change in cytoplasmic Ca^{2+} levels. Thus, it could be suggested that ADP acts on adenylyl cyclase through $G\alpha_{i2}$ and that this particular G-protein does not provide sufficient concentrations of free $\beta\gamma$ complexes for PLC activation in response to adrenaline or ADP stimulation. Alternatively, PLC-β_2 and -β_3 may not be expressed in platelets. Further studies are needed to answer these points.

Since the activation of $G\alpha_{i2}$ by ADP cannot account alone for its aggregatory effect, how then can the stimulation of ADP receptor(s) result in mobilization of intracellular Ca^{2+} stores? In contrast to most aggregating agents which mobilize Ca^{2+} through D-*myo*-inositol 1,4,5-triphosphate, the signal transduction pathways of ADP are poorly understood [3]. ADP induces an increase in intracellular Ca^{2+} in a unique manner, involving both activation of non-selective cation channels [27] and mobilization of internal stores by a still unidentified pathway. Studies of the mechanism of action of the anti-thrombotic drugs ticlopidine and clophidogrel, which are specific inhibitors of ADP-induced platelet aggregation [28], have shown that in the case of rat platelets, the inhibition of the increase in intracellular Ca^{2+} is due to blockade of the mobilization of internal stores [29]. Although we did not find the same effect in human volunteers [30], this discrepancy could arise from the fact that the doses of drugs given to the animals were very much higher than those permissible in humans. These drugs do inhibit the ADP receptor-induced activation of G-proteins [14]. However, whether the Ca^{2+}-mobilizing effect of ADP receptor stimulation is a G-protein-dependent effect remains to be established. Studies of patients with selective deficiency of ADP-induced platelet aggregation [31–32], who have reduced numbers of binding sites for 2MeSADP, a specific P_{2T} agonist, should be of interest in this respect, and we are currently investigating such a patient with our system [33].

CONCLUSIONS

Our results confirm that ADP-induced platelet activation is partly due to a G-protein-coupled receptor, which interacts with the $G\alpha_{i2}$ heterotrimeric G-protein. This may explain how ADP inhibits adenylyl cyclase. On the other hand, the absence of activation of the G_q pathway raises the question of the mechanism of Ca^{2+} mobilization and the subsequent aggregation process for which, as in case of adrenaline, inhibition of adenylyl cyclase is not sufficient.

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- **Cover Illustration:** The active site of the Theta class glutathione transferase from *Lucilia cuprina*. The position of the bound glutathione is shown relative to the Ser-9 hydroxy group. Reproduced from Board et al. (1995) Biochem. J. **311**, 247–250.

Identification of the Pertussis Toxin-Sensitive G Proteins in Platelets, Megakaryocytes, and Human Erythroleukemia Cells

By Alison G. Williams, Marilyn J. Woolkalis, Mortimer Poncz, David R. Manning, Alan M. Gewirtz, and Lawrence F. Brass

Guanine nucleotide-binding regulatory proteins, or G proteins, mediate the interaction of agonist receptors on the platelet surface with phospholipase C and adenylyl cyclase. To better understand this process, we have used several approaches to identify which G proteins are present in platelets, normal human megakaryocytes, and human erythroleukemia (HEL) cells, a leukemic cell line with megakaryocytic features. Because platelet and HEL cell responses to thrombin are inhibited by pertussis toxin, we have focused upon the members of the G_i family, whose α subunits can be ADP-ribosylated by that toxin. Western blots with antisera specific for $G_{i\alpha}$ demonstrated the presence in both platelets and HEL cells of the three best-described forms of this protein: $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$. Based upon immunoprecipitation studies with [^{35}S]-methionine-labeled HEL cells, their relative abundance appears to be $G_{i\alpha2} \gg G_{i\alpha3} > G_{i\alpha1}$. A HEL cell cDNA library screened with the $G_{i\alpha}$ antisera produced clones encoding $G_{i\alpha2}$ and $G_{i\alpha3}$ that had sequences similar to those reported from other sources. $G_{i\alpha}$-specific probes created from these cDNA clones confirmed the presence of mRNA encoding $G_{i\alpha2}$ and $G_{i\alpha3}$ in both platelets (by Northern blotting) and megakaryocytes (by in situ hybridization). Thus the pertussis toxin substrates that have previously been detected in platelets and HEL cells are shown to be members of the $G_{i\alpha}$ family, all of which are candidates for interaction with receptors for thrombin and other agonists.

THE ABILITY OF thrombin to stimulate phosphoinositide hydrolysis by phospholipase C and to inhibit cAMP formation by adenylyl cyclase in human platelets is believed to be mediated by one or more pertussis toxin-sensitive guanine nucleotide-binding regulatory proteins, or G proteins.[1-11] However, although such proteins are known to exist in platelets, there continues to be uncertainty about their identities. For example, inhibition of cAMP formation is generally ascribed to the G protein referred to as G_i, but cloning studies in cells other than platelets have shown that there are at least three forms of G_i, with α subunits designated $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$.[12,13] These proteins are 85% to 95% homologous with each other, but they differ in their tissue distribution and may serve different functions. Which of them is present in platelets and whether other pertussis toxin-sensitive G proteins are also present is unknown.

Many of the same considerations apply to the G protein or proteins that mediate agonist-induced phosphoinositide hydrolysis by phospholipase C. This G protein is often referred to as "G_p," but to date no single G protein has been shown to be uniquely capable of mediating the interaction between agonist receptors and phospholipase C. It is possible that one of the members of the $G_{i\alpha}$ family serves as G_p and regulates phospholipase C in cells in which agonist-induced phosphoinositide hydrolysis is inhibited by pertussis toxin, but this remains to be established.

In previous studies the pertussis toxin-sensitive G protein α subunits present in platelets were detected by incubating permeabilized platelets or platelet membranes with the toxin and ^{32}P-NAD.[5,14-17] When analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), a single band of radioactivity was observed that had an apparent mass of approximately 41 Kd, a size that is compatible with any of the known $G_{i\alpha}$ types. At least two radiolabeled proteins within this band could be resolved by isoelectric focusing.[18] Limited digestion of each of these with trypsin or staphylococcal V8 protease gave rise to identical arrays of radiolabeled fragments.[18] This suggests that the pertussis-toxin substrates in platelets are highly homologous to each other but leaves open the question of whether they are distinct but related proteins or a single protein that has undergone a post-translational modification affecting its isoelectric point.

Many of these observations about pertussis toxin, G proteins, and the interaction of thrombin receptors with adenylyl cyclase and phospholipase C also apply to human erythroleukemia (HEL) cells. HEL cells are a human leukemic cell line that has been shown to synthesize proteins that are characteristic of the megakaryocyte/platelet lineage, including membrane glycoproteins Ib, IIb, and IIIa and the secretory protein, platelet factor 4.[19-21] In addition, HEL cells, like platelets, express thrombin receptors that are coupled to phospholipase C and adenylyl cyclase via one or more pertussis toxin-sensitive G proteins, making them a useful model for some aspects of platelet function.[22,23] In the present studies we have used immunologic and molecular biologic approaches to identify and to determine the relative abundance of the G protein α subunits in platelets, HEL cells, and megakaryocytes that are substrates for pertussis toxin. The results suggest that all of the previously noted

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pertussis-toxin substrates in platelets are members of the $G_{i\alpha}$ family.

METHODS

Antibodies. The peptides used to prepare $G_{i\alpha}$-specific antisera for these studies were selected from published, full-length, predicted amino-acid sequences of the three fully described forms of $G_{i\alpha}$. The regions that were selected were either common to all three members of the family or specific for just one. The sequences and approximate locations of the peptides are shown in Fig 1, together with the number used to refer to each antiserum. Several of these have been described previously.[24] The peptide used in generating antiserum 8730 was coupled to keyhole limpet hemocyanin (KLH) using glutaraldehyde.[25] In all other cases a cysteine residue was added to the amino terminus of the peptide to permit coupling to KLH using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester.[26] The polypeptide conjugates were injected at multiple intradermal sites on female New Zealand white rabbits.[27]

Antiserum 1398 was prepared from a peptide whose sequence is common to $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$. Antiserum 8730 was prepared from a peptide sequence present in $G_{i\alpha1}$ and $G_{i\alpha2}$ but differing by two amino residues from the corresponding sequence in $G_{i\alpha3}$. Antisera 3646 and 1521 were prepared from peptides specific for $G_{i\alpha1}$ and $G_{i\alpha2}$ and are theoretically identical to those previously described by Goldsmith et al.[28] A sample of the $G_{i\alpha1}$-specific antiserum LD/2 prepared by Goldsmith et al[28] was kindly provided to us by Dr A. Spiegel and was used for comparison. Antiserum 1518 was prepared from a peptide specific for $G_{i\alpha3}$. The predicted specificities of all of these antisera were confirmed by immunotransfer blotting using recombinant $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ that were synthesized using vectors in *Escherichia coli* supplied by Drs M. Linder and A. Gilman[29] (University of Texas Health Sciences Center, Dallas) (Fig 2).

Membrane preparation. Platelet membranes were prepared as described.[18] HEL cell membranes were prepared from cells growing in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS).[22] To prepare the membranes, the HEL cells were resuspended at greater than 10^7/mL in 10 mmol/L Tris-HCl, 1 mmol/L $MgCl_2$, 5 mmol/L EDTA, 10 mmol/L benzamidine, 20 μg/mL leupeptin, 0.1% (vol/vol) aprotinin and 0.9 mmol/L PMSF at pH 8.0 and then disrupted by 20 to 40 strokes in a Dounce-style homogenizer at 4°C. Greater than 95% cell disruption was confirmed by microscopic examination. Any cells that remained intact were removed from the lysate by low-speed sedimentation (250*g* for 5 minutes). A membrane pellet was prepared by centrifugation at 31,400*g* for 15 minutes and then resuspended in 1 mmol/L EGTA with 50 mmol/L HEPES, pH 7.0, at a final protein concentration of 1 to 2 mg/mL.

Immunotransfer blotting. The membrane proteins (50 to 100 μg) from platelets and HEL cells were resolved by SDS-PAGE and transferred to nitrocellulose for immunostaining.[30,31] The $G_{i\alpha}$-selective antisera were used at a 1:100 dilution. Antibody binding was detected by incubation with biotinylated goat antirabbit IgG followed by avidin-horseradish peroxidase (Vector Laboratories, Inc, Burlingame, CA) and visualized with 4-chloronaphthol.

Immunoprecipitation and two-dimensional analysis of [^{35}S]-methionine-labeled HEL cells. HEL cells were incubated overnight in RPMI 1640 containing 5 μmol/L methionine, 10% dialyzed FCS, and 50 μCi [^{35}S]-methionine/mL in the absence or presence of 100 ng/mL pertussis toxin. Membranes (50 μg) were prepared,[24] boiled in 0.5% SDS with 1 mmol/L dithiothreitol (DTT), and then diluted in buffer to yield 150 mmol/L NaCl, 50 mmol/L sodium phosphate, pH 7.2, 2 mmol/L EDTA, 1 mmol/L DTT, 10 mg/mL aprotinin, 0.5% SDS, 1% sodium deoxycholate, 1% Triton X-100, 5 mmol/L NaF, 2 mmol/L $Na_4P_2O_7$, 2 mmol/L Na_3VO_4, and 0.2 mg/mL leupeptin. Immunoprecipitation was performed as described by Carlson et al.[24] To reduce nonspecific binding of [^{35}S]-labeled proteins in immunoprecipitates, samples were incubated with nonimmune serum (1:100) at 4°C for 1 hour, followed by Pansorbin (Calbiochem) for 2 hours. The Pansorbin and bound protein were removed by centrifugation. Antisera were added to the supernatants at dilutions ranging from 1:8 to 1:100 and incubated at 4°C overnight. The incubation was continued for 2 hours after the addition of Protein A-Sepharose. Precipitates were isolated by centrifugation, washed three times by resuspension and centrifugation in 150 mmol/L NaCl, 50 mmol/L sodium phosphate, pH 8.0, 2 mmol/L EDTA, and 0.5% Triton X-100. Two-dimensional PAGE analysis was performed by a procedure modified from O'Farrell.[32,33] The precipitates were prepared for isoelectric focusing by suspension in 2.5% Lubrol-PX, 2% Bio-Lytes 5/7 (BioRad, Richmond, CA), 0.1% sodium deoxycholate, 5 mmol/L K_2CO_3, 40 mmol/L DTT, and

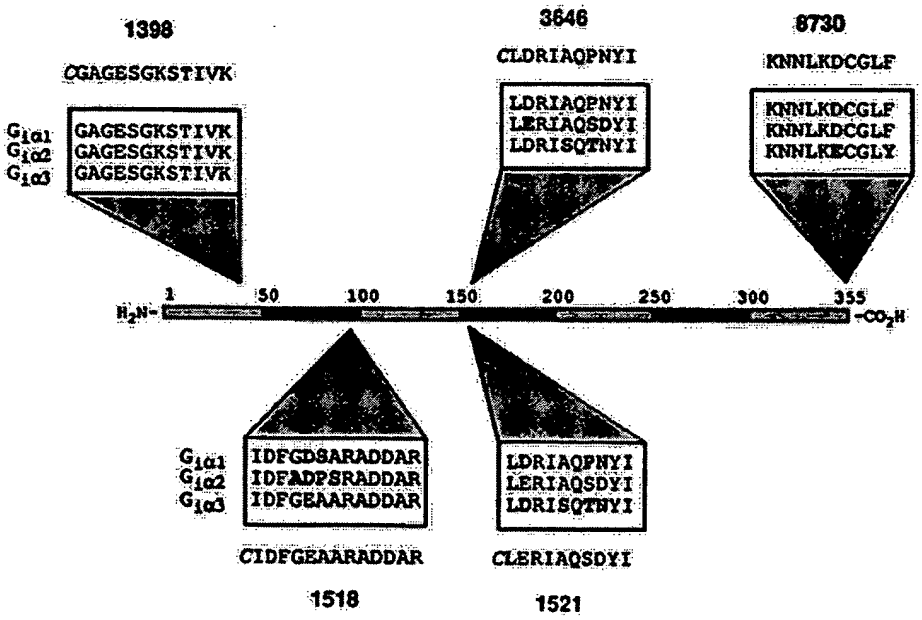

Fig 1. Location/sequence of peptides used to develop the anti-$G_{i\alpha}$ antibodies. The figure shows the regions of a generic $G_{i\alpha}$ recognized by peptide-directed antibodies specific for $G_{i\alpha1}$, $G_{i\alpha2}$, and/or $G_{i\alpha3}$. The numbers in bold type identify each antiserum. The peptides shown outside the boxes refer to those synthesized and conjugated to keyhole limpet hemocyanin. The peptides shown within the boxes represent the analogous sequences of amino acids present within the listed subunits, with differences from the immunizing peptides portrayed in bold type.

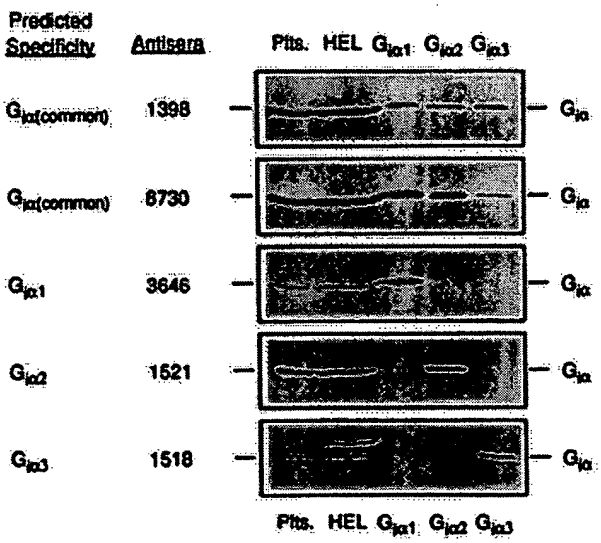

Fig 2. Immunotransfer blotting of platelet and HEL cell membranes and recombinant $G_{i\alpha}$. Partially purified preparations containing recombinant $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ and membranes (100 μg) from platelets and HEL cells were subjected to SDS-PAGE followed by immunotransfer blotting. The peptide-directed antisera employed had predicted specificities for sequences common to all forms of $G_{i\alpha}$ (1398, 8730) or unique to a single form of $G_{i\alpha}$ (3646, 1521, 1518); the peptide sequences and approximate locations are noted in Fig 1.

9.5 mol/L urea. The isoelectric focusing gels contained 2% Lubrol PX, 4% Bio-Lytes 5/7, 9.5 mol/L urea, and 4% acrylamide; isoelectric focusing was performed at 800 V for 16 hours using 50 mmol/L NaOH as the catholyte and 30 mmol/L H_3PO_4 as the anolyte. Typically the pH gradients were linear from pH 5.3 to 6.9. The second dimension was discontinuous SDS-PAGE.

Isolation of cDNA clones for HEL Cell $G_{i\alpha}$ subunits. Total cellular RNA was obtained from HEL cells using the method of Chirgwin et al.[34] Poly (A)$^+$ RNA was isolated by chromatography on oligo (dT)-cellulose.[35] A cDNA expression library in the vector λgt11 was used as previously described.[21] Phage containing $G_{i\alpha}$ subunit cDNA were identified by screening a lawn of transformed *E coli* Y1090 with antiserum 1398, which recognizes $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ (Figs 1 and 2). Plaques reacting with the antibody were identified using biotinylated goat antirabbit antiserum and an avidin-horseradish peroxidase complex to detect the secondary antibody (Vector Laboratories, Inc).[19]

Characterization of $G_{i\alpha}$ clones. Phage DNA from antibody-positive clones was purified and digested with *Eco*R1.[21] The cDNA inserts were isolated by electroelution and subcloned into M13mp18 or M13mp19 bacteriophage[36] and transfected into JM107 bacteria to prepare single-stranded DNA in both orientations. cDNA insert orientation and homology were determined by annealing single-stranded DNA from different isolates and digesting the hybrids with S1 nuclease (Bethesda Research Laboratories).[37] The nucleotide sequences of the cDNA were obtained using the dideoxy chain termination technique of Sanger et al[38] and the universal M13 primer (Bethesda Research Laboratories).[39] The complete sequence of $G_{i\alpha2}$ cDNA was obtained by preparing a series of overlapping deletion mutants using Bal 31 endonuclease.[37] The sequence for the $G_{i\alpha3}$ clone was obtained using overlapping 19-bp oligonucleotide primers that were designed using published sequences.[40]

Northern blot analysis of HEL and platelet total RNA. Total RNA from HEL cells, platelets, and white blood cells (WBCs) was prepared by the acid guanidinium-phenol-chloroform method.[41] The HEL cells were grown in RPMI with 10% FCS. Human platelets and white cells were isolated from freshly obtained samples of whole blood obtained from normal donors and anticoagulated with acid-citrate-dextrose.[24] After sedimenting the red cells, platelets were collected from the upper two thirds of the platelet-rich plasma (PRP), taking care to avoid the buffy coat, and then washed. A white cell-enriched fraction was obtained from the buffy coat. Prepared in this fashion, light microscopy revealed approximately 1 white cell per 2,000 cells in the "platelet" preparation.

Northern blotting was performed as described.[42] Typically, 10 to 15 μg of total RNA was run on a 1% agarose-formaldehyde gel in 0.2 mol/L MOPS pH 7.0, 50 mmol/L sodium acetate, and 1 mmol/L EDTA. The RNA was then transferred to Zetabind nitrocellulose filters (Micro Filtration Products) in 10-times concentrated SSC (150 mmol/L NaCl and 15 mmol/L sodium citrate, pH 7.0) and dried. The filter was washed for 30 minutes in 0.2 × SSC with 0.5% SDS at 68°C. Prehybridization was carried out at 68°C for 1 hour with 50 μg/mL sonicated salmon sperm DNA and 10 μg/mL poly rA in a buffer containing 0.5 mol/L sodium phosphate, pH 7.5, 7% SDS, 1% bovine serum albumin (BSA), and 1 mmol/L EDTA.

Specific cDNA probes were made from restriction fragments of the 3' untranslated regions of the two HEL cell cDNA clones identified as $G_{i\alpha2}$ and $G_{i\alpha3}$. The $G_{i\alpha2}$-specific probe was a 752 bp *Kpn* 1/*Eco*RI fragment. The $G_{i\alpha3}$ probe was a 299 bp *Sph* 1/*Eco*RI fragment. A full-length cDNA for $G_{i\alpha1}$ prepared from a human brain cDNA library was a gift from Drs P. Bray and M. Nirenberg.[43] A specific probe from this cDNA was made from a *Sca*I/*Eco*RI 254-bp restriction fragment composed entirely of the 3' untranslated region. The platelet factor 4 probe was a 408-bp cDNA insert.[19] A 1.8-kB probe for the LFA-1$_\beta$ subunit was provided by Dr T. Springer (referred to as "3.1.1" in reference).[44] The probes were radiolabeled to a specific activity of 10^7 cpm/mL by the calf thymus random primer method[45] using either radiolabeled α-[^{32}P]-deoxycytidine alone or α-[^{32}P]-deoxycytidine with α-[^{32}P]-deoxyadenosine simultaneously (Amersham, Arlington Heights, IL).

Megakaryocytes: Isolation and in situ hybridization. Human megakaryocytes were obtained from normal donors, enriched by counterflow centrifugal elutriation, and placed into short-term suspension culture.[46,47] After 24 hours, aliquots of cells were fixed for 30 minutes at room temperature in 4% paraformaldehyde containing 5 mmol/L $MgCl_2$, washed twice in diethylpyrocarbonate-treated water, and then stored in 70% ethanol at 4°C. Cells were subsequently deposited onto glass slides by cytocentrifugation for analysis. Megakaryocytes were identified using standard morphological criteria of size, nuclear configuration, and nuclear/cytoplasmic ratio[48] supplemented by immunochemical identification with a mouse monoclonal antibody (MoAb) to platelet glycoproteins IIb and IIIa.[49]

In situ hybridization was carried out with the cDNA probes described above for the Northern blotting studies oligolabeled with biotin-11-dUTP (Bethesda Research Laboratories) as previously described.[47] Briefly the fixed megakaryocytes were rehydrated for 10 minutes in phosphate-buffered saline (PBS) containing 5 mmol/L $MgCl_2$, then washed sequentially in triethanolamine (0.1 mol/L), acetic anhydride (0.25% vol/vol in triethanolamine), PBS-5 mmol/L $MgCl_2$, and 0.1 mol/L glycine in 0.2 mol/L Tris-HCl (pH 7.4). Slides were then rinsed in 2 × SSC and finally placed in 2 × SSC, 45% formamide (vol/vol; Fluka Chemical, Ronkonkoma, NY) at 65°C for 10 minutes. The slides were removed from this solution and placed on a slide warmer at 42°C. Labeled probe, dissolved in hybridization cocktail (100 ng probe/mL) containing 45% formamide (Amresco, Solon, OH) and 1 mg/mL tRNA, was heat denatured at 95°C for 10 minutes, after which time 30 μL was layered over the cells. Hybridization was allowed to proceed for 18

hours at 37°C, after which the slides were washed with decreasing concentrations of SSC followed by 0.1 mol/L, Tris-HCl, 0.1 mol/L NaCl, pH 7.5, containing 3% BSA (crystalline grade, Sigma Chemical, St Louis, MO). cDNA-RNA hybrids were detected by the sequential addition of streptavidin, biotinylated alkaline phosphatase, and the chromogens nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolylphosphate using a DNA Detection Kit (Bethesda Research Laboratories). Positive reactions consisted of a purple-blue to deep-brown-colored precipitate in the cell's cytoplasm. Each experiment included biotin-11-dUTP–labeled pBR322 (Promega, Madison, WI) as a negative control and a biotin-11-dUTP–labeled cDNA encoding human β-actin[20] as a positive control.

RESULTS

Identification of platelet and HEL cell G protein α subunits by immunotransfer blotting. Peptide-directed antisera that are specific for each of the known members of the $G_{i\alpha}$ family were prepared by injecting rabbits with the KLH-conjugated polypeptides shown in Fig 1. The predicted specificities of the antisera were based upon comparison of the amino acid sequences of the various G_α in the region encompassed by the peptide. The actual specificities of the antisera were determined by immunotransfer blotting with recombinant α subunits (Fig 2). Based upon this analysis, antiserum 1398 recognizes all three forms of $G_{i\alpha}$, while antisera 3646, 1521, and 1518 are specific for $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$, respectively. Antiserum 8730 also recognizes all three forms of $G_{i\alpha}$ but recognizes $G_{i\alpha3}$ less well than $G_{i\alpha1}$ and $G_{i\alpha2}$. This difference is consistent with the presence within the peptide used to prepare antiserum 8730 of two amino acids that are different from those present in the corresponding position of $G_{i\alpha3}$ (Fig 1). The specificity of antisera 3646, 1521, and 1518 for their respective forms of $G_{i\alpha}$ was reaffirmed by their lack of reactivity with the alternative forms of $G_{i\alpha}$ even when the recombinant proteins were added at 10 times the amounts in Fig 2 (not shown).

Figure 2 also includes the results that were obtained when the antisera were allowed to react with the proteins from platelet and HEL cell membranes. Each of the antisera gave a positive reaction with both types of membranes, suggesting that all three of the $G_{i\alpha}$ family members are present in platelets and HEL cell.

Two-dimensional electrophoresis of HEL cell immunoprecipitates. All of the known $G_{i\alpha}$ family members contain the cysteine residue at which ADP-ribosylation by pertussis toxin normally occurs.[12,13] Our previous studies showed that the pertussis-toxin substrates in platelets can be resolved by two-dimensional electrophoresis into at least two proteins that have the same apparent mass (~41 Kd) but different isoelectric points.[18] Based upon limited proteolysis, those proteins appeared to be highly homologous with each other, but it was not possible to identify them with certainty. For the studies shown in Fig 3, membrane proteins were immunoprecipitated from [^{35}S]-methionine–labeled HEL cells using the $G_{i\alpha}$-specific antisera. When indicated, the HEL cells were also incubated overnight with pertussis toxin prior to membrane preparation, so that the pertussis toxin-sensitive G proteins would be ADP-ribosylated as well as [^{35}S]-methionine labeled. The concentration of toxin that was used, 100 ng/mL, is sufficient to completely inhibit HEL cell responses to thrombin and fully ADP-ribosylate the available pertussis toxin substrates in HEL cells.[51] Immunoprecipitates from the radiolabeled HEL cells were analyzed by two-dimensional electrophoresis and autoradiography.

The results of the immunoprecipitation studies are shown in Fig 3. The radiolabeled proteins representing $G_{i\alpha}$ are identifiable by (1) their characteristic migration just below actin at an apparent mol wt of ~41 Kd and (2) their acidic shift in isoelectric point (rightward as shown in the figure) when the cells were incubated with pertussis toxin. With antiserum 1398, which recognizes $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$, at least three distinct radiolabeled species are visible. One of these, which is consistenily the most intensely radiolabeled, migrates immediately beneath actin in the absence of pertussis toxin. This protein was selectively immunoprecipitated with the $G_{i\alpha2}$-specific antiserum, 1521, identifying it as $G_{i\alpha2}$. A less intensely radiolabeled, slightly more basic protein was selec-

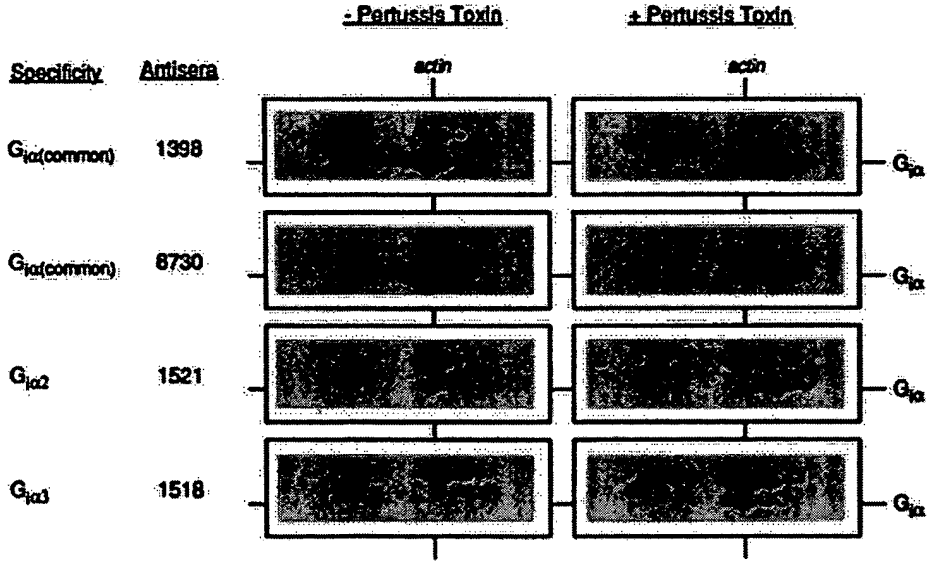

Fig 3. Two-dimensional analysis of $G_{i\alpha}$ in HEL cell immunoprecipitates. Membranes (50 μg) prepared from HEL cells metabolically labeled with [^{35}S]-methionine in the absence or presence of pertussis toxin were immunoprecipitated with antisera directed against common epitopes of $G_{i\alpha}$ (1398, 8730) or antisera specific for $G_{i\alpha2}$ (1521) or $G_{i\alpha3}$ (1518). The immunoprecipitates were subjected to isoelectric focusing followed by SDS-PAGE and visualized by autoradiography. Actin, which was present in the immunoprecipitates as a contaminant and which has an apparent mass slightly greater than the $G_{i\alpha}$, served as an internal marker.

tively immunoprecipitated with the $G_{i\alpha3}$-specific antiserum, 1518, suggesting that it is $G_{i\alpha3}$. The locations of these two proteins after ADP ribosylation is similar to that previously observed by us for ^{32}P-ADP-ribosylated proteins isolated from platelet membranes incubated with ^{32}P-NAD and pertussis toxin,[18] suggesting that those proteins were also $G_{i\alpha2}$ and $G_{i\alpha3}$. Additional support for this conclusion was obtained by using antisera 1521 and 1518 to immunoprecipitate ^{32}P-ADP-ribosylated proteins from platelet membranes. Each antiserum immunoprecipitated a single radiolabeled species whose relative position on the two-dimensional autoradiogram was similar to those shown in the right half of Fig 3 (not shown).

In addition to the two proteins identified as $G_{i\alpha2}$ and $G_{i\alpha3}$, a third protein was seen in the HEL cell immunoprecipitates with antisera 1398 and 8730 that had an isoelectric point more basic than either $G_{i\alpha2}$ or $G_{i\alpha3}$ (Fig 3). This was the least intensely radiolabeled of the three distinguishable proteins. Based upon its immunoprecipitation by antisera 1398 and 8730 and not by the $G_{i\alpha2}$ and $G_{i\alpha3}$-selective antisera 1521 and 1518, it is tentatively identified as $G_{i\alpha1}$. This conclusion is supported by the observed mobility of purified brain $G_{i\alpha1}$ on two-dimensional gels reported by Goldsmith et al.[52] However, the $G_{i\alpha1}$-specific antiserum, 3646, which might have confirmed this identification, failed to immunoprecipitate detectable amounts of any of the radiolabeled HEL cell proteins.

Identification and sequencing of $G_{i\alpha}$ clones from an HEL cell cDNA library. An HEL cell cDNA library previously used to obtain full-length clones for platelet factor 4 and membrane glycoproteins IIb and IIIa[19-21] was screened with antiserum 1398, which recognizes all three forms of $G_{i\alpha}$. From 40,000 plaques tested, 10 positive clones were isolated. Nine of these were identified as $G_{i\alpha2}$ by restriction mapping and/or sequence analysis. The remaining clone encoded $G_{i\alpha3}$. No clones expressing $G_{i\alpha1}$ were found among those purified from the HEL cell library.

The two longest clones of each type were sequenced completely. The $G_{i\alpha2}$ clone is 2081 nucleotides long and 100% homologous in its predicted coding region to a human lymphocyte $G_{i\alpha2}$ cDNA clone (Fig 4).[40] It is, however, several hundred nucleotides longer in the 3′ untranslated region. Minor differences in the 5′ and 3′ untranslated regions of the HEL cell and lymphocyte clones may represent actual differences in these regions due to genetic drift.

The $G_{i\alpha3}$ clone is 1469 nucleotides in length and also demonstrates close homology to human lymphocyte $G_{i\alpha3}$,[40] although the HEL cell clone is slightly truncated, missing the 5′ untranslated region and the first three amino acids (Fig 5).

Detection of mRNA encoding $G_{i\alpha}$ in platelets. The HEL cell cDNA clones shown in Figs 4 and 5 were used to develop probes that could be used to detect messenger RNA (mRNA) encoding $G_{i\alpha2}$ and $G_{i\alpha3}$ in platelets. To minimize cross-reactivity between different $G_{i\alpha}$ subtypes, restriction fragments were prepared from the 3′ untranslated region of each. The 752-bp *KpnI/EcoRI* fragment from $G_{i\alpha2}$ recognized a band migrating at approximately 2.4-kB in both HEL cell and platelet RNA (Fig 6A). The 299-bp *SphI/EcoRI* fragment from $G_{i\alpha3}$ hybridized with a species at 1.65 Kb in

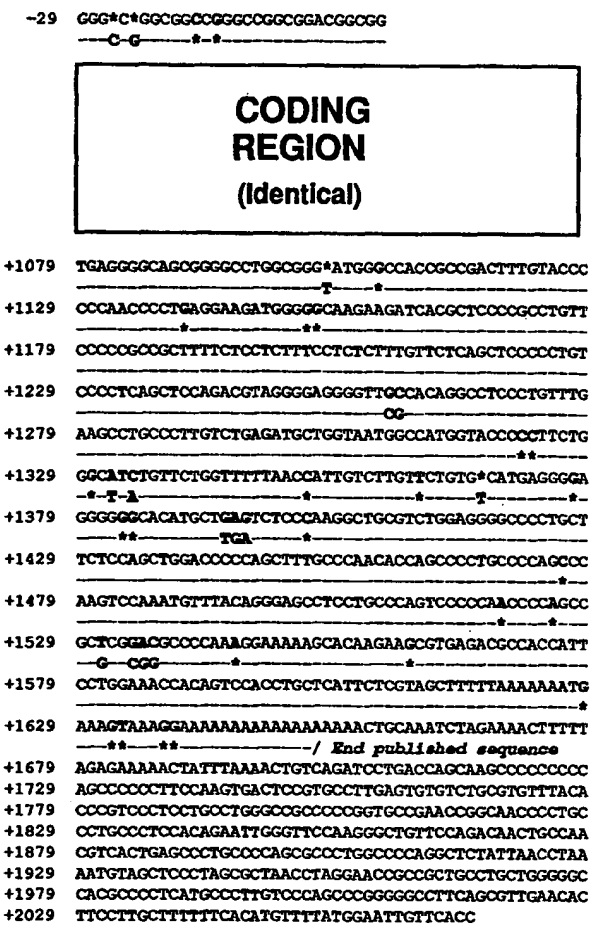

Fig 4. Sequence of HEL cell $G_{i\alpha2}$. Schematic of the HEL cell $G_{i\alpha2}$ clone (top line) shown in comparison to a human lymphocyte $G_{i\alpha2}$ clone (bottom line).[40] An (*) denotes the absence of a base in a particular sequence; –, identity between the HEL cell and lymphocyte sequences. Differences are shown in bold. The coding regions, shown in the boxed areas, are identical.

both platelets and HEL cells (Fig 6B). In contrast, neither platelet nor HEL cell RNA hybridized consistently with a 254-bp *ScaI/EcoRI* fragment composed of the entire 3′ untranslated region of a human brain $G_{i\alpha1}$ clone.[43]

Human platelets contain only relatively small amounts of RNA, which represents residual megakaryocyte RNA. Several approaches were used to ensure that the $G_{i\alpha}$ RNA detected in platelets by Northern blotting was actually from platelets and not due solely to contamination from the WBCs that are invariably present in platelet preparations. First, a probe that recognizes RNA encoding the platelet-specific protein, platelet factor 4, was used to confirm the presence of platelet RNA. Second, a probe against the β subunit of the neutrophil protein, LFA1, was used to detect white-cell RNA.[44] Comparison of the relative amounts of PF4, $LFA1_\beta$, and $G_{i\alpha2}$ RNA detectable in typical platelet preparations with the amounts detectable in deliberately white cell-enriched RNA preparations confirmed the presence in the platelet preparation of amounts of $G_{i\alpha2}$ in excess of that which could be attributed to contamination by white cell RNA (not shown).

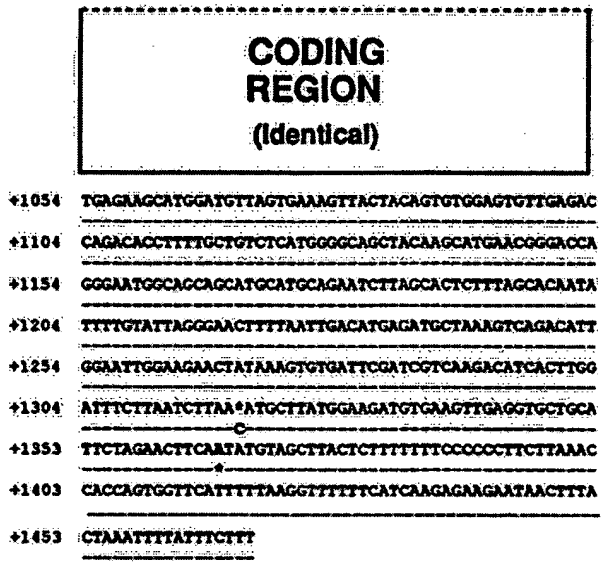

Fig 5. Sequence of HEL cell $G_{i\alpha 2}$. Schematic of the HEL cell $G_{i\alpha 2}$ clone (top line) shown in comparison to a human lymphocyte $G_{i\alpha 2}$ clone (bottom line).[40] The HEL cell clone is missing the equivalent of three amino acids at the amino (5') terminus, but the coding region (boxed area) is otherwise identical. An (*) denotes the absence of a base in a particular sequence; –, identity between the HEL cell and lymphocyte sequences. Differences are shown in bold.

In situ hybridization studies with megakaryocytes. Further confirmation that mRNA encoding $G_{i\alpha 2}$ and $G_{i\alpha 3}$ is present in platelets was obtained by examining megakaryocytes by in situ hybridization techniques that allowed direct visualization and identification of individual cells. For these studies the fragments from the 3' untranslated regions of the HEL cell $G_{i\alpha 2}$ and $G_{i\alpha 3}$ cDNA clones and the brain $G_{i\alpha 1}$ cDNA clone were oligolabeled with biotin-11-dUTP and incubated with megakaryocytes that had been fixed with paraformaldehyde. Hybridization of the probes to the megakaryocyte RNA was detected using the streptavidin-alkaline phosphatase color reaction. A positive signal is indicated by the appearance of purple-brown precipitate in the cytoplasm of the cell, the density of which is related to abundance of the mRNA.[47,53] The results in Fig 7 show that megakaryocytes react strongly with the $G_{i\alpha 2}$ probe and, to a lesser extent, the $G_{i\alpha 3}$ probe. The $G_{i\alpha 1}$ probe gave a result that was equivocal or negative compared to the pBR322 control.

DISCUSSION

The interaction of thrombin receptors with adenylyl cyclase and phospholipase C in human platelets and HEL cells is thought to be mediated by one or more G proteins with α subunits that are substrates for pertussis toxin. This conclusion is based largely on the evidence from studies of cell function which are reviewed elsewhere.[54] However, there remains uncertainty about the number of pertussis toxin-sensitive G proteins present in platelets and the identity of the particular G proteins that stimulate phosphoinositide hydrolysis ("G_p") and inhibit cAMP formation ("G_i"). In our previous studies, we found that platelets contain at least two highly homologous pertussis-toxin substrates with the same mol wt, ~41 Kd but slightly different isoelectric points.[18] Both of these appeared to be coupled to thrombin receptors, since preincubation of the cells with thrombin-inhibited subsequent ADP-ribosylation of the proteins, possibly by altering the configuration necessary for interaction with pertussis toxin.[15,18,55]

When permeabilized platelets[5] or intact HEL cells[51] are incubated with pertussis toxin, there is a close correlation between the extent of ADP-ribosylation and the loss of thrombin's ability to stimulate phosphoinositide hydrolysis

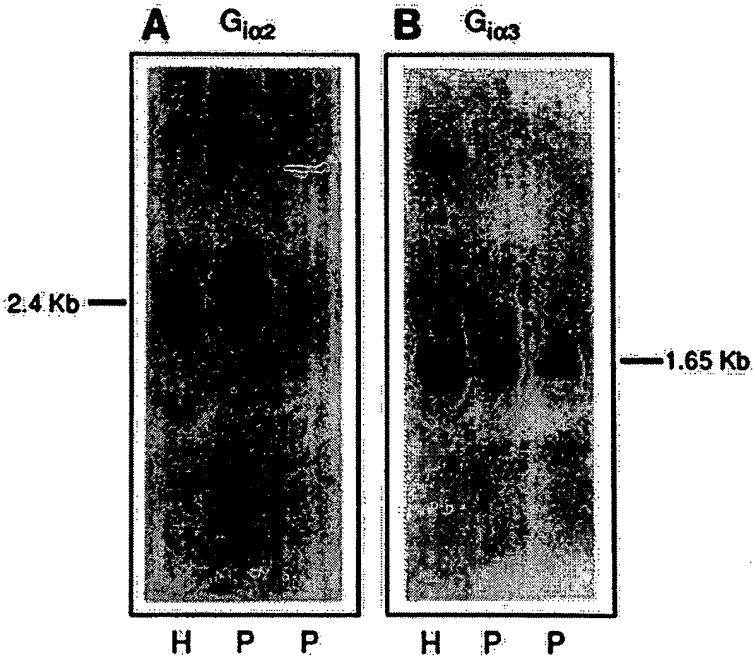

Fig 6. Northern blots of platelets and HEL cell using the $G_{i\alpha 2}$ and $G_{i\alpha 3}$ 3' probes. RNA from HEL cells (H) and platelets (P) was prepared and analyzed as described in Methods and probed with radiolabeled $G_{i\alpha}$-specific fragments created from the 3' untranslated regions of the HEL cell $G_{i\alpha 2}$ (A) and $G_{i\alpha 3}$ (B) clones.

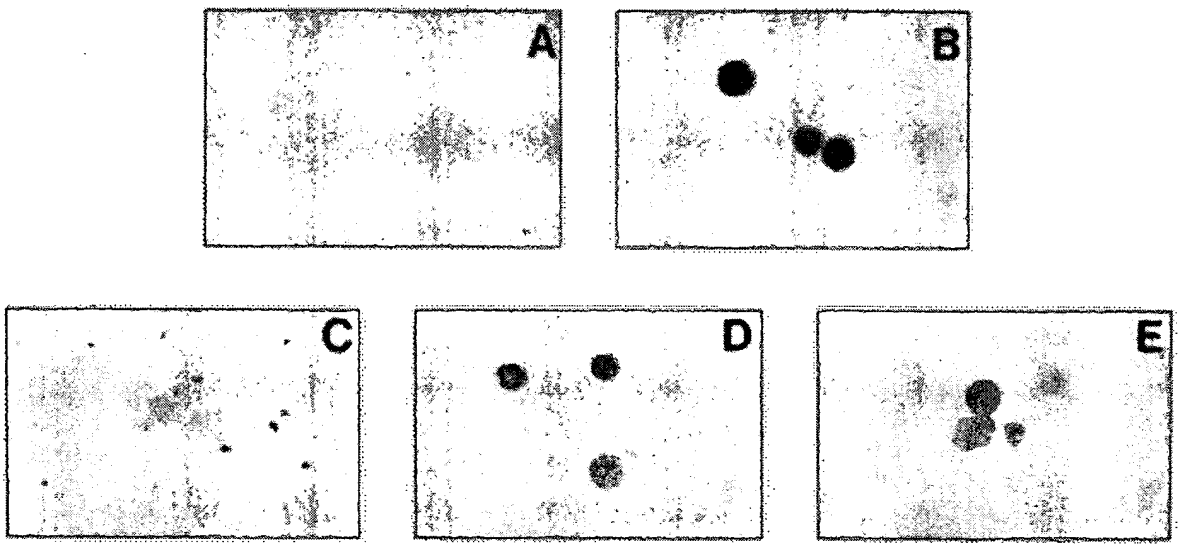

Fig 7. Megakaryocytes: In situ hybridization with G_{α}-selective probes. Representative photomicrographs (black and white rendering of original color pictures) of normal human megakaryocytes probed for expression of $G_{\alpha i1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$ mRNA by in situ hybridization with biotin-11-dUTP–labeled cDNA probes (panels C, D, and E). A positive signal is indicated by a purple-brown precipitate in the cytoplasm of the cell. The appearance of cells after hybridization with biotin-11-dUTP–labeled probes for pBR322 (negative control) and β-actin (positive control) is shown in panels A and B, respectively.

and to inhibit cAMP formation. Collectively these observations suggest that thrombin receptors interact with at least two G proteins whose α subunits have an apparent mass of approximately 41 Kd and similar susceptibility to pertussis toxin. The goal of the present studies was to identify these proteins.

The results suggest that all of the pertussis toxin-sensitive G protein α subunits that have been detected in platelets and HEL cells are members of the $G_{i\alpha}$ family. Incubation of platelet and HEL cell membranes with peptide-directed antisera of established specificity revealed $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ by Western blotting. When the same antisera were used to immunoprecipitate proteins extracted from [^{35}S]-methionine–labeled HEL cells, three radiolabeled proteins could be identified that had the expected mol wt and that underwent the appropriate shift in isoelectric point upon ADP-ribosylation by pertussis toxin. The relative mobility of these proteins was consistent with that observed previously by Goldsmith et al[52] for purified brain $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$. Assuming that the three proteins incorporate [^{35}S]-methionine to approximately the same extent and are equally immunoprecipitable by the "common" antiserum 1398, the relative abundance of the proteins is $G_{i\alpha2} \gg G_{i\alpha3} > G_{i\alpha1}$. Based upon similarity of size and isoelectric behavior, it is likely that the two ^{32}P-ADP-ribosylated proteins noted in our earlier studies were $G_{i\alpha2}$ and $G_{i\alpha3}$.

This conclusion is compatible with recent observations by two other groups of investigators. Nagata et al[56] isolated two pertussis-toxin substrates from cholate extracts of platelets. Based upon limited sequencing, the more abundant protein appeared to be $G_{i\alpha2}$. The less abundant was tentatively identified as either $G_{i\alpha1}$ or $G_{i\alpha3}$. Similarly, Crouch et al[57] detected a prominently immunoreactive 40-Kd protein in platelets using a peptide-directed, $G_{i\alpha2}$-selective antiserum, LE/3.[28] LE/3 is theoretically equivalent to our antiserum 1521. Those authors did not have access to a $G_{i\alpha3}$-selective antibody and obtained a negative result with the $G_{i\alpha1}$-selective LD/2 prepared by Goldsmith et al.[28] Although we have obtained a sample of LD/2 and found that it gives a weakly positive result with platelet and HEL cell membranes when used at high concentration, the essential message appears to be the same from both studies: platelets and HEL cells contain more immunologically recognizable $G_{i\alpha2}$ than $G_{i\alpha1}$. On the other hand, we have been unable to confirm the observation by Crouch et al[57] of a 38-Kd G_o-like platelet protein using any of the G_o-directed antisera employed in this or an earlier study,[24] including the C-terminal peptide $G_{i\alpha}$ antiserum 8730 that is theoretically equivalent to the AS/7 antiserum used by Crouch et al[57] (Fig 2).

The relationship between the $G_{i\alpha2}$ and $G_{i\alpha3}$ present in platelets and HEL cells and the corresponding proteins present in other cells was explored by isolating and sequencing clones encoding $G_{i\alpha2}$ and $G_{i\alpha3}$ from a HEL cell cDNA library. Based upon predicted amino-acid analysis, the coding regions of the HEL cell proteins appears to be identical with those from other sources. Tissue or species-specific differences in the 3' untranslated regions of the clones, which are not unexpected, were exploited to create non-cross-reactive cDNA probes. These probes were used to confirm that RNA encoding $G_{i\alpha2}$ and $G_{i\alpha3}$ is present in platelets (by Northern blotting) and megakaryocytes (by in situ hybridization). The ability to directly visualize megakaryocytes during the in situ hybridization studies confirmed the source of the reactivity.

Thus the immunologic and molecular biological studies suggest that platelets and HEL cells contain all three known members of the $G_{i\alpha}$ family. Our failure to detect $G_{i\alpha1}$ in the HEL cell cDNA expression library by antibody screening, in

Table 1. G Protein α Subunits in Platelets

	Size (Kd)	Toxin	Phosphorylated?	Enzyme	Function
$G_{s\alpha}$[61]	45	Cholera	—	Adenylyl cyclase	↑cAMP
$G_{i\alpha2} \gg G_{i\alpha3} > G_{i\alpha1}$	40-41	Pertussis	No[24]	Adenylyl cyclase	↓cAMP
				Phospholipase C?	↑IP_3/DG?
$G_{z\alpha(plt)}$[24]	41	—	Yes[24]	?	?

The identities and possible roles of the G proteins currently known to exist in platelets. The data for the $G_{i\alpha}$ family are discussed in this report.

platelet RNA by Northern analysis, or in megakaryocytes by in situ hybridization suggests that it is, in fact, the least abundant of the three proteins in these cells. An alternative explanation, which is that the positive Western blot seen with the $G_{i\alpha1}$-selective antiserum shown in Fig 2 was due to cross-reactivity with $G_{i\alpha2}$ or $G_{i\alpha3}$, seems less likely. We were unable to detect such cross-reactivity even when the concentration of recombinant $G_{i\alpha2}$ and $G_{i\alpha3}$ were increased 10-fold over the amounts shown in Fig 2. Furthermore, the same Western blot result with recombinant $G_{i\alpha}$ and membranes from platelets and HEL cells was obtained with a second $G_{i\alpha1}$-selective antiserum directed against the same peptide sequence.[28]

All of the G protein α subunits currently known to exist in platelets are listed in Table 1. The pertussis-toxin substrates are members of the $G_{i\alpha}$ family. All of these have an apparent mass of 40 to 41 Kd. An additional pertussis-toxin substrate with an apparent mass of 39-Kd has been reported[9] but has not been a general finding.[5,14-17] The assignment of phospholipase C regulation to one or more of the members of the $G_{i\alpha}$ family is speculative. Based upon the inhibitory effects of a $G_{i\alpha}$-selective antiserum, $G_{i\alpha2}$ has been implicated in the regulation of adenylyl cyclase in platelets,[58] but equivalent data are not yet available for phospholipase C. There is also, as yet, no explanation for the observed ability of thrombin to interact with both phospholipase C and adenylyl cyclase, while epinephrine is limited to adenylyl cyclase. Data from a number of laboratories suggest that thrombin receptors and epinephrine (α_2-adrenergic) receptors are differentially coupled to the pertussis toxin-sensitive G proteins in platelets,[15,18,55,57] but the precise basis for this differentiation is still unknown. In an earlier study we demonstrated, based upon the criterion of the ability of an agonist to inhibit ADP-ribosylation of G proteins coupled to its receptors, that both epinephrine and thrombin are coupled to what are now shown to be $G_{i\alpha2}$ and $G_{i\alpha3}$.[18] This suggests that the differences in the functional responses of platelets to thrombin and epinephrine are probably not attributable solely to differences in the interaction of their receptors with these two G proteins.

In addition to the members of $G_{i\alpha}$ family, platelets contain at least two forms of G_{α} that are not substrates for pertussis toxin. The first of these is $G_{s\alpha}$, the α subunit of the G protein that stimulates adenylyl cyclase in platelets in response to agents such as PGI_2 and PGE_1.[59-61] $G_{s\alpha}$ exists as two subfamilies with different apparent masses.[62,63] Based upon radiolabeling with cholera toxin, platelets and HEL cells contain at least the 45-Kd form of $G_{s\alpha}$.[61] The second pertussis toxin-insensitive G protein in platelets has the same apparent mol wt as the members of the $G_{i\alpha}$ family but is antigenically related to $G_{z\alpha}$ rather than $G_{i\alpha}$.[24] $G_{z\alpha}$ is a putative G protein α subunit that was originally cloned from human retina and rat brain.[64,65] It is only 60% homologous with $G_{i\alpha}$ and has no known function. Based upon immunotransfer blotting with $G_{z\alpha}$-specific antisera, the amount of the $G_{z\alpha}$-like protein ("$G_{z\alpha(plt)}$") in platelets probably lies between $G_{i\alpha3}$ and $G_{i\alpha2}$.[24] How similar $G_{z\alpha(plt)}$ is to $G_{z\alpha}$ remains to be determined. Although not a substrate for pertussis toxin, $G_{z\alpha(plt)}$ is phosphorylated during platelet activation.[24] The effects of this phosphorylation and the role of $G_{z\alpha(plt)}$ are unknown.

In conclusion, the currently available evidence suggests that one or more pertussis toxin-sensitive G proteins helps to regulate phospholipase C and adenylyl cyclase in both platelets and HEL cells. The data from the present studies demonstrate that both types of cells contain at least two members of the $G_{i\alpha}$ family that are candidates for this role. Which G protein is involved in which reaction and the role of pertussis toxin-insensitive G proteins such as G_z remain to be determined.

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Antiplatelet Therapy

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The major clinical indication for antiplatelet therapy has been the prevention of arterial thrombosis. Arterial thrombi are composed of predominantly platelets formed under conditions of elevated shear stress at sites of atherosclerotic vascular injury and disturbed blood flow. Aspirin, the prototype antiplatelet agent, has been in clinical use as an antithrombotic for almost a half century. However, clinical trials have exposed the limitations of aspirin, and there has been considerable recent progress in the development of more effective antiplatelet agents. These newer agents are rationally based on interrupting specific sites in the sequence of platelet activation. Inhibitors of the initial step of platelet adhesion remain experimental. Inhibitors of specific platelet agonist-receptor interactions include antithrombins, thromboxane A_2 receptor antagonists, and adenosine diphosphate (ADP) receptor blockers including ticlopidine and clopidogrel. Inhibitors of arachidonic acid metabolism and thromboxane A_2 include omega-3 fatty acids, aspirin and other nonsteroidal antiinflammatory drugs that inhibit cyclooxygenase, and thromboxane synthase inhibitors. The clinical efficacy of many of these agents may be limited by their actions, which are restricted to single, specific platelet receptors or metabolic pathways. Global interruption of the final step of platelet aggregation can be achieved with monoclonal antibodies and RGD (arginine-glycine-aspartic acid) analogs that block ligand binding to the platelet glycoprotein IIb/IIIa complex. Initial clinical trials with these novel agents have demonstrated superior efficacy in preventing reocclusion and restenosis following coronary angioplasty and atherectomy. *Am J Med.* 1996;101:199–209.

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Arterial thrombosis frequently causes acute and irreversible damage or infarction of target organs, most notably the heart and brain, leading to death or permanent disability. Therefore, while considerable progress has been made in the treatment and rehabilitation of survivors of arterial thrombosis, the major impetus is to prevent the occurrence of these events. To this end, the rapid recent development of more effective antithrombotic agents has proceeded in parallel with elucidation of modifiable risk factors for atherosclerotic cardiovascular disease, which is characteristically the substrate for arterial thrombosis.

ARTERIAL AND VENOUS THROMBOSIS

The pathophysiology of arterial thrombosis differs in some basic respects from that of venous thrombosis.[1,2] Venous thrombi tend to form in the absence of underlying vascular damage. They generally result from the combined effects of reduced blood flow (stasis) and activated coagulation proteins. Venous thrombi are composed predominantly of red cells enmeshed in fibrin, hence the term "red thrombi." Consistent with this pathophysiology, patients with inherited hypercoagulable states (thrombophilias), which are characterized by activation of the coagulation cascade and increased fibrin formation, typically have venous rather than arterial thrombotic complications. Furthermore, anticoagulants (heparin, warfarin) that inhibit fibrin generation, not antiplatelet agents, have been the established treatment for venous thromboembolism. In contrast, arterial thrombi tend to form under conditions of elevated wall shear stress at sites of vascular injury and disturbed blood flow. They develop most commonly in atherosclerotic vessels. Arterial thrombi ("white thrombi") are composed predominantly of platelets and relatively little fibrin or red cells. Therefore, it has been generalized that antiplatelet agents rather than anticoagulants should be the primary strategy for the treatment and prevention of arterial thrombosis.

The major clinical indications for the antiplatelet agents to be reviewed in this article are, in fact, coronary, cerebrovascular and peripheral arterial thrombosis. Nevertheless, it must be emphasized that the pathophysiological and clinical distinctions between arterial and venous thrombosis are not absolute. First, platelet activation and fibrin formation are inseparable and interdependent events in the processes of hemostasis and thrombosis: thrombin

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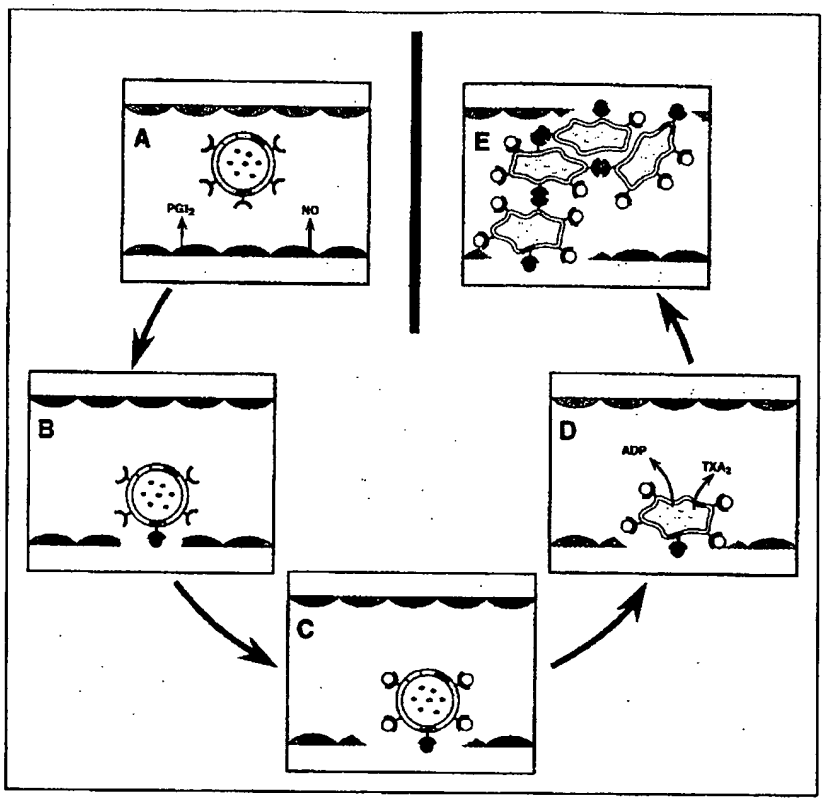

Figure 1. Mechanisms of platelet activation at a site of vascular injury. **(A)** Release of platelet inhibitory products of endothelial cells, including prostacyclin (PGI_2) and nitric oxide (NO), maintains quiescent state of platelets traversing intact vessels. **(B)** Disruption of endothelium exposes thrombogenic subendothelial vessel wall constituents (eg, collagen); platelet adhesion to this site is mediated by von Willebrand factor (vWf) binding to its platelet receptors localized in membrane glycoprotein (Gp) Ib. **(C)** Other platelet activators (eg, thrombin, epinephrine) bind to their specific platelet receptors. **(D)** Platelets activated by these stimuli degranulate and undergo the release reaction, releasing ADP and thromboxane A_2 (TXA_2); ADP and TXA_2 bind to their respective platelet receptors to further amplify the platelet activation process. **(E)** Platelet aggregation is mediated by fibrinogen or vWf binding to platelet membrane GpIIb/IIIa, a functional heterodimeric receptor which is expressed on the surfaces of only activated platelets.

is a potent activator of platelets and, reciprocally, activated platelets provide a surface for enhanced thrombin generation. Second, both venous and arterial thrombi are to different degrees composed of both platelets and fibrin, and they are in continuous states of dynamic remodeling, undergoing propagation, organization, embolization, lysis, and rethrombosis, resulting in constantly changing compositions.[1] Therefore, the categorization of venous clots as fibrin thrombi and arterial clots as platelet thrombi is an oversimplification. Finally, although their efficacy relative to more established antiplatelet (aspirin) therapy has not been demonstrated, anticoagulants are effective in preventing acute myocardial infarction in patients with unstable angina or previous myocardial infarction[3] and, to a lesser extent, in preventing stroke in patients with cerebrovascular disease.[4] Conversely, the use of particularly the newer antiplatelet agents should be further investigated for the prevention of venous thromboembolism as well as arterial thrombosis.

Aspirin, the prototype antiplatelet agent, has been in clinical use as an antithrombotic for almost a half century.[5] Dr. L. L. Craven, an otorhinolaryngologist in private practice, reported in the *Mississippi Valley Medical Journal* that none of more than 8,000 patients treated with aspirin in an uncontrolled trial in the early 1950s developed acute myocardial infarction.[6] Until recently, aspirin was the only effective antiplatelet agent available in clinical practice. Aspirin remains the "gold standard" antiplatelet agent, largely because of its relative safety and extremely low cost. However, clinical trials have exposed the limitations of aspirin, providing the impetus for a recent explosion of work to develop more effective antiplatelet agents. In contrast to aspirin, the initial use of which was largely empirical, these newer agents are rationally based on our understanding of platelet function. Therefore, a brief review of the basic mechanism of platelet activation is necessary to appreciate current and potential therapeutic targets of antiplatelet intervention.

MECHANISM OF PLATELET ACTIVATION

As shown in **Figure 1A**, the monolayer of endothelial cells that normally lines the intima of the entire circulatory tree is a thromboresistant surface that secretes potent, locally-acting platelet inhibitory products. The two best characterized endothelium-derived platelet inhibitors are prostacyclin (PGI_2) and nitric oxide (NO), which inactivate platelets by raising intraplatelet cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) levels, respectively. These autocoids are also secreted into the vessel wall to relax smooth muscle. Their net effects, therefore, are to preserve blood fluidity. At a site of vascular injury, shown in Figure 1B, thromboresistant endothelium is disrupted and prothrombotic subendothelial vessel wall constituents (eg, collagen) are exposed to blood. Circulating platelets recognize these intimal breaks and undergo the process of "adhesion," in which a carpet of platelets become anchored to the vessel wall. The ligand for adhesion is von Willebrand factor (vWf), which binds to specific platelet receptors localized in membrane glycoprotein (Gp) Ib.

A variety of other platelet stimuli (eg, collagen, thrombin, epinephrine, serotonin) can simultaneously bind to their specific platelet surface receptors (Figure 1C), and they function in concert to trigger a cascade of intracellular reactions that lead to the activation and subsequent aggregation of platelets. Fluid shear stress can also directly activate platelets. Activated platelets undergo the release reaction (Figure 1D), secreting preformed substances (eg, adenosine diphosphate [ADP]) from their intracellular storage granules. Platelet activation also induces phospholipase A_2 (PLA_2)-mediated hydrolysis of free arachidonic acid (AA) from membrane phospholipid pools (**Figure 2**). Free AA is then rapidly metabolized by cyclooxygenase (COX) to the labile prostaglandin endoperoxides, PGG_2 and PGH_2, and then by thromboxane synthase (TxS) to thromboxane A_2 (TXA_2). Released ADP and PGH_2/TXA_2 bind to their respective platelet receptors to further amplify the platelet activation process. Platelet activation (and vasoconstriction) by TXA_2 is antagonized in the presence of intact endothelium by platelet inhibitory and vasodilatory PGI_2, which is produced in endothelial cells through prostacyclin synthase (PS) from either endothelium-derived or platelet-derived endoperoxides.

Finally, activated and degranulated platelets attach to each other in the process of aggregation to form an occlusive thrombus at the site of vascular damage (Figure 1E). The ligand for platelet aggregation is fibrinogen (or vWf in higher shear stress regions of the circulation). The platelet receptor for these aggregating ligands is exposed only on the surfaces of activated platelets by the complexing of GpIIb and GpIIIa to form the functional heterodimeric GpIIb/IIIa complex. The binding of fibrinogen or vWf to their platelet GpIIb/IIIa receptors is mediated by the tripeptide amino acid sequence, arginine-glycine-aspartic acid (abbreviated "RGD"), a motif which is present on both of these adhesive molecules.

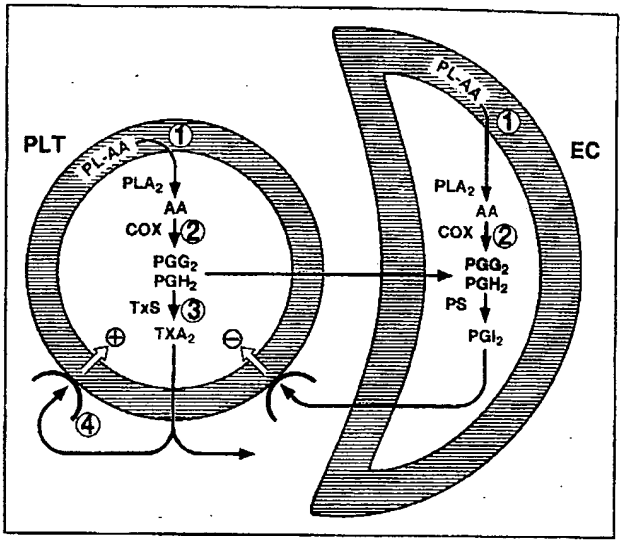

Figure 2. Platelet (PLT)-endothelial cell (EC) interactions mediated by arachidonic acid (AA) metabolites. Activators of each cell type induce the phospholipase A_2 (PLA_2)-mediated hydrolysis of free AA from membrane phospholipid (PL) pools. Arachidonic acid is converted in each cell type by cyclooxygenase (COX) to prostaglandin endoperoxides, PGG_2 and PGH_2. Endoperoxides are metabolized to thromboxane A_2 (TXA_2) by thromboxane synthase (TxS) in platelets and to PGI_2 by prostacyclin synthase (PS) in endothelial cells. TXA_2 binds to platelet receptors to stimulate platelet activation; PGI_2 binds to separate platelet receptors to inhibit platelet activation. (These eicosanoids likewise exert opposing actions on vascular tone, TXA_2 causing vasoconstriction and PGI_2 causing vasorelaxation.) Endothelial cells can also utilize platelet-derived endoperoxides as substrate for PS. Antiplatelet agents have targeted AA mobilization from membrane PL pools (1), AA oxygenation by COX (2), thromboxane synthase (3) or TXA_2 receptors (4).

Each of these steps of platelet activation has been targeted in the development of antiplatelet agents (**Figure 3**).

ANTIPLATELET AGENTS

Stimuli of Platelet cAMP and cGMP

Intravenous infusion of prostacyclin may have transient beneficial effects in coronary artery disease,[7] peripheral arterial disorders,[8] and thrombotic thrombocytopenic purpura.[9] However, systemic use is limited by its potent vasoactive effects and extreme lability. Regional administration of prostacyclin prevents extracorporeal platelet consumption and reduces the risk of platelet embolization and bleeding in cardiopulmonary bypass, charcoal hemoperfusion, and hemodialysis.[10] Attempts have been

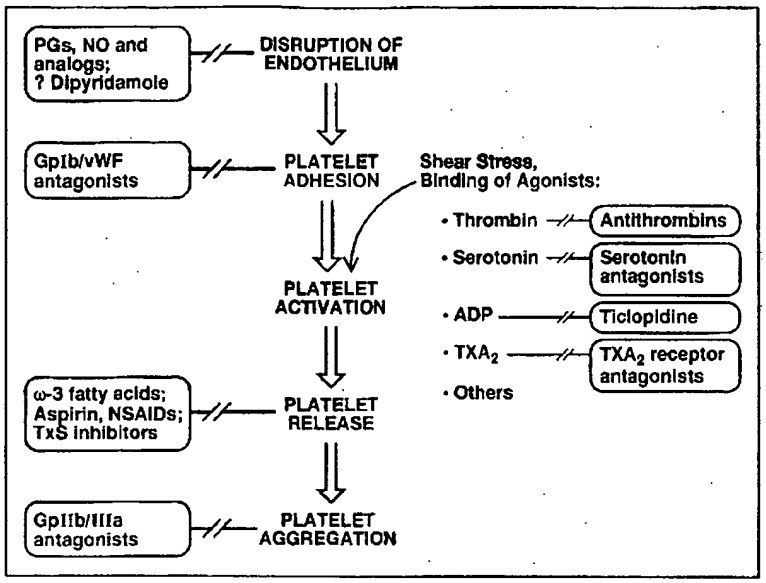

Figure 3. Steps of platelet activation targeted in the development of antiplatelet agents. PGs = prostaglandins; NO = nitric oxide; GpIb = glycoprotein Ib; vWf = von Willebrand factor; NSAIDs = nonsteroidal antiinflammatory drugs; TxS = thromboxane synthase; GpIIb/IIIa = glycoprotein IIb/IIIa; TXA_2 = thromboxane A_2.

TABLE

Clinical Efficacy of Aspirin, Ticlopidine and Dipyridamole

Indication	Aspirin	Ticlopidine	Dipyridamole
Coronary artery disease			
Primary prevention of MI	+/–		
Stable angina	+		
Unstable angina	+	+	
Secondary prevention of MI	+		–
Coronary artery bypass graft	+	+	–
Acute occlusion after angioplasty	+	+	–
Restenosis after angioplasty	–	–	–
Other cardiac disease			
Mechanical or high risk tissue valves	+W		+W
Atrial fibrillation (nonrheumatic)	+		
Cerebrovascular disease			
Primary prevention of stroke	–		
Stroke or recurrent TIA after TIA or minor stroke	+	+	–
Secondary prevention of stroke (after major stroke)		+	–
Stroke or restenosis after endarterectomy	–		
Shunt thrombosis	+		
Peripheral vascular disease	+	+	+A

Clinical efficacy designations represent generalizations based on weight of evidence in the literature.
Table does not indicate number or powers of studies or specific doses or regimens of antiplatelet agents.
Further information can be found in more detailed reviews (3, 4, 26, 78, 105) and original publications.
+ = effective; – = ineffective; +/– = equivocal; +A = effective when used with aspirin; +W = effective when used with warfarin.
MI = myocardial infarction; TIA = transient ischemic attack.

made to develop more stable synthetic analogues of prostanoids with less adverse hemodynamic actions.[11] Iloprost, one such compound that can also be administered orally,[12] exerts prolonged clinical benefit in patients with limb ischemia.[13] However, paradoxical platelet hyperreactivity and hypercoagulability have been noted during and after iloprost infusion.[14]

Dipyridamole[15] has been suggested to act as an antiplatelet drug by several possible mechanisms. It directly stimulates prostacyclin synthesis,[16] potentiates the platelet inhibitory action of prostacyclin,[17] inhibits phosphodiesterase to raise platelet cyclic AMP levels,[18] and blocks the uptake of adenosine into vascular and blood cells, thereby causing accumulation of this platelet-inhibitory and vasodilatory

compound in the thrombotic microenvironment.[15,19] However, since these effects may not occur at therapeutically achievable concentrations of the drug, the mechanism of action of dipyridamole remains unclear.[15,20] At therapeutic doses, dipyridamole does not prolong the bleeding time[21] or inhibit ex vivo platelet aggregation.[22] Clinical trials (**Table**) have failed to demonstrate antithrombotic efficacy of dipyridamole used alone in any clinical setting.[15] Furthermore, dipyridamole does not add to the beneficial effects of aspirin in the secondary prevention of myocardial infarction,[23] the prevention of stroke in patients with prior transient ischemic attacks (TIA) or stroke,[24,25,26] or maintenance of coronary artery bypass graft patency.[27] Dipyridamole may add to the beneficial effect of aspirin in preventing the progression of peripheral occlusive arterial disease.[28] It may also enhance the efficacy of warfarin in preventing systemic embolism from mechanical heart valve prostheses,[29,30] although direct benefit and risk comparisons between aspirin and dipyridamole as additions to warfarin have not been established.[31] Dipyridamole does not increase bleeding risk, even when combined with anticoagulants,[32] but its side effects include dose-related gastrointestinal symptoms, headache, and, rarely with oral dosing, a coronary "steal" phenomenon. FitzGerald[15] concluded that, particularly in view of its variable absorption kinetics, cost and side effects, the emerging consensus does not support the use of dipyridamole as an antiplatelet agent.

Organic nitrates and other NO donors have platelet inhibitory and vasodilatory properties.[33,34] However, preliminary results of human trials have not demonstrated increased efficacy of linsidomine (SIN-1), a NO donor, compared with isosorbide nitrate and diltiazem hydrochloride in unstable angina and coronary angioplasty.[35]

Inhibitors of Platelet Adhesion

Agents that block the interaction of vWf with its platelet membrane GpIb receptor should inhibit adhesion as well as the subsequent downstream cascade of platelet activation events, including the secretion of platelet mitogens into the vessel wall and aggregation. This therapeutic approach could involve anti-vWf or anti-GpIb monoclonal antibodies, recombinant vWf fragments that bind to GpIb and thus block its interaction with intact vWf, and aurin tricarboxylic acid (ATA), which binds to vWf and prevents its interaction with platelet GpIb.[36,37] There is presently no experience with the use of these agents in humans. However, the promise of this antiplatelet strategy is illustrated by experimental experience with an inbred strain of bleeder pigs with severe vWf deficiency, comparable to humans with homozygous von Willebrand disease (vWd). Severe vWd pigs have markedly reduced spontaneous and diet-induced atherosclerosis, and are protected from coronary thrombosis following vascular injury.[38,39] Administration of anti-vWf monoclonal antibodies to normal pigs likewise prevents experimental coronary thrombosis.[40,41] Enthusiasm for this attractive target for potent antiplatelet agents is tempered by concerns that platelet adhesion inhibitors would also significantly interfere with normal hemostasis, as illustrated by the spontaneous bleeding diathesis of vWd and platelet GpIb deficiency (Bernard-Soulier syndrome).

Inhibitors of Platelet Agonist-Receptor Interactions

A theoretical limitation of inhibitors of individual platelet agonist-receptor interactions is their ability to block the initiation of only one of several pathways of platelet activation. Perhaps surprisingly, therefore, some of these agents have proven to be experimentally and clinically effective as antiplatelet therapy.

The most potent platelet stimulus is thrombin. Direct antithrombins (eg, hirudin) or inhibitors of thrombin generation should therefore not only prevent fibrin formation but also inhibit thrombin-induced platelet activation[42,43]; the latter could be directly inhibited by thrombin receptor antagonist peptides. The important role of antithrombins as antiplatelet agents has been established in some experimental models of thrombosis.[44,45] However, it is unknown whether these findings can be extended to the treatment of human arterial thrombosis and, if so, whether the optimal antiplatelet regimen will also be the optimal anticoagulant regimen of thrombin inhibitors.[42]

Of the platelet-derived agonists, thromboxane A_2 receptor antagonists (discussed below) and ADP receptor blockers have received the most attention. Ticlopidine[46] and its analog, clopidogrel, are thienopyridine derivatives that exert their antiplatelet action by inhibiting the binding of ADP to its platelet receptors[47] and thereby inhibiting ADP-induced platelet aggregation. These drugs may also blunt platelet aggregation in response to other stimuli, the actions of which are mediated by ADP released from endogenous platelet granule pools.[48,49] Earlier observations that ticlopidine inhibits fibrinogen binding to its platelet Gp IIb/IIIa receptors[50] probably reflect a downstream platelet activation event following ADP receptor blockade. Notably, unlike aspirin, ticlopidine also inhibits platelet aggregation in response to shear stress.[51]

Maximal antiaggregatory action is noted only after oral administration of ticlopidine and cannot be re-

produced in vitro with pharmacologic concentrations of the drug. There is a 3- to 5-day delay in achieving full antiplatelet activity following the drug's administration; the bleeding time does not become maximally prolonged until 5 to 6 days after starting it.[52] Therefore, the antiplatelet activity of ticlopidine is probably mediated by an in vivo metabolite.[49] Action persists for 4 to 8 days after drug discontinuation, consistent with the lifetime of platelets in the circulation, indicating an irreversible antiplatelet effect.

The clinical efficacy of ticlopidine is summarized in the Table. It may be the most effective antiplatelet agent currently available for stroke prevention.[4] The Ticlopidine versus Aspirin Stroke Study (TASS) showed that ticlopidine (500 mg daily) was superior to aspirin (1,300 mg daily) for prevention of stroke in patients with a history of TIA or minor stroke.[53] At the 3-year study endpoint, the ticlopidine group had a 21% reduction in stroke or stroke death compared with the aspirin group. The efficacy advantage of ticlopidine was even greater during the first year of treatment, the period of highest risk for stroke recurrence. Subgroup analysis at 5 years of follow-up also demonstrated the superiority of ticlopidine for prevention of recurrent TIAs.[54] Nevertheless, since many patients in the TASS study were "aspirin failures" at the time of their qualifying events, some uncertainty remains regarding the relative efficacy of ticlopidine versus aspirin for preventing stroke or TIA in patients not previously treated with aspirin.[55] The Canadian-American Ticlopidine study found ticlopidine superior to placebo in the secondary prevention of stroke,[56] but ticlopidine and aspirin have not yet been directly compared for this indication.

Clinical trials have also demonstrated the efficacy of ticlopidine in preventing death or myocardial infarction in patients with unstable angina,[57] preventing coronary artery bypass graft occlusion,[58] and treating intermittent claudication[59] and diabetic retinopathy.[60] However, direct comparisons of ticlopidine with aspirin for these indications have yet to be reported.

Despite the convincing data from TASS showing somewhat greater efficacy of ticlopidine, aspirin continues to be the treatment of choice for stroke prevention because of ticlopidine's adverse effects, particularly severe neutropenia in about 1% of patients. Neutropenia tends to occur early and is usually, but not always,[61] reversible after cessation of therapy. More common side effects include rash and diarrhea. Therefore, ticlopidine (at a recommended dose of 250 mg twice daily) is now generally reserved for patients with TIA or stroke who have aspirin intolerance or fail aspirin therapy. The cost-effectiveness of ticlopidine, which was recently analyzed,[62] depends on the country in which it is used.

Inhibitors of Arachidonic Acid Metabolism and Thromboxane A_2

Several antiplatelet strategies have been based on interruption of platelet TXA_2 synthesis and/or action. Drugs can block platelet arachidonic acid (AA) mobilization, AA oxygenation by COX, thromboxane synthase, and TXA_2 receptors (see Figure 2).

The reduced incidence of atherosclerotic cardiovascular disease in Greenland Eskimos has been attributed to their marine lipid-rich diets.[63] Cod liver oil supplementation of the atherogenic diets of hyperlipidemic swine,[64] as well as dietary fish supplementation of human populations,[65,66] has a protective effect on the development of coronary atherosclerosis. The predominant polyunsaturated fatty acids in the Eskimo diet are the omega-3 fatty acids, so designated because the first double bond is located on the third carbon atom from the methyl (omega) terminus. A major omega-3 fatty acid in fish oils is eicosapentaenoic acid (EPA). The antithrombotic alterations in platelet-vascular interactions attributed to fish oils are due to incorporation of EPA into cell membrane phospholipids, leading to competition between EPA and AA as substrates for COX. The COX metabolism of AA in platelets produces the platelet-activating and vasoconstrictor TXA_2, whereas the product of EPA metabolism is TXA_3, which is biologically inert. In vascular cells, however, both PGI_2 and PGI_3, COX products of AA and EPA metabolism, respectively, possess comparable platelet inhibitory and vasodilatory properties. Omega-3 fatty acids thus produce a favorable shift in the balance of these eicosanoids toward an antithrombotic effect. Large and potentially unpalatable doses (>10 g EPA daily) of medicinal fish oils are required to alter cell membrane fatty acid contents that simulate those attained with Eskimo diets.[67]

Individuals consuming "Eskimo diets" have a mild bleeding tendency, prolonged bleeding times, mild thrombocytopenia, and abnormalities in ex vivo platelet aggregability. In addition to their antiplatelet effects, fish oils may have other protective actions on atherosclerotic cardiovascular disease,[68] including hypolipidemic, antiinflammatory, and rheologic effects. The most extensive use of omega-3 fatty acids as antiplatelet therapy has been in preventing restenosis following coronary angioplasty. Meta-analyses of earlier trials demonstrated a small to moderate beneficial effect,[69,70] but a recent randomized trial of 8 g per day of omega-3 fatty acids for 6 months after angioplasty failed to prevent restenosis.[71]

Aspirin (acetylsalicylic acid) irreversibly inactivates COX by acetylating serine residues at position 529, leading to inhibition of platelet TXA_2 synthesis. Aspirin likewise blocks the production of prostacyclin, the major COX metabolite of AA in vascular endothelial cells, creating an "aspirin dilemma"[72] and incompletely successful attempts to devise a "platelet-specific" aspirin regimen. Aspirin has a half-life of only 20 minutes in the systemic circulation. However, since acetylation irreversibly inhibits COX activity and platelets have only a rudimentary capacity to synthesize new, unacetylated protein, exposure of platelets to a single dose of aspirin impairs their function for their remaining lifetimes (7 to 10 days) in the circulation.

Inhibition of TXA_2 formation does not affect the initial process of platelet adhesion, but does block TXA_2-mediated release and hence partially inhibits the final step of aggregation. Platelet aggregation in response to stimuli that induce release independent of TXA_2 (eg, thrombin, shear stress) is not significantly impaired by aspirin. In contrast to ticlopidine, the inhibitory actions of aspirin on platelet TXA_2 production are rapid, with maximal effects attained within 15 to 30 minutes of ingesting a dose as low as 81 mg.[73] Reduced ex vivo platelet aggregability persists for about 4 to 7 days after a single dose of aspirin,[74] although the prolonged bleeding time returns to normal within 24 to 48 hours. This discrepancy may be due to the emergence from the marrow into the circulation of a sufficient cohort of uninhibited platelets to restore normal in vivo hemostasis (measured by the bleeding time) even before ex vivo platelet function is completely normalized.

Roth and Calverley[75] and Patrono[76] have recently reviewed the mechanism of action, pharmacology, clinical efficacy, and safety of aspirin. The recently published report of the Antiplatelet Trialists' Collaboration[77] provided meta-analyses combining patient data across all methodologically sound trials of prophylactic antiplatelet therapy available for review before March 1990. The most widely tested antiplatelet regimen was aspirin, 75 to 325 mg daily. It was concluded that antiplatelet agents given to patients at increased risk for occlusive vascular disease reduced all-cause mortality, vascular mortality, vascular events, and nonfatal myocardial infarction. As indicated in the Table, aspirin is probably effective for primary and secondary prevention of myocardial infarction, stable and unstable angina, coronary artery bypass graft patency, mechanical or high-risk tissue valves (when added to warfarin), nonrheumatic atrial fibrillation (although warfarin is superior), and prevention of stroke or recurrent TIA in patients with TIA. Aspirin is also possibly effective in peripheral vascular disease and to prevent shunt thrombosis, spontaneous abortion in patients with lupus anticoagulant, and complications of pregnancy-induced hypertension,[78] although a large, recently completed randomized trial challenged its efficacy for the latter indication.[79]

Aspirin does have important theoretical limitations. Experimental models have shown that catecholamines, which are important endogenous modulators of platelet reactivity,[80] can overcome the inhibitory effects of aspirin on coronary thrombosis.[81] Furthermore, aspirin does not inhibit platelet responses to some potent platelet stimuli, such as shear stress levels attained in stenosed arteries.[82] It is somewhat surprising, in fact, that aspirin provides as much protection against arterial thrombosis as numerous clinical trials have demonstrated, raising the possibility that aspirin's therapeutic benefit is mediated by mechanisms in addition to COX inhibition.[83]

Nonaspirin nonsteroidal antiinflammatory drugs (NSAIDs) inhibit platelet function by reversible inhibition of COX. Therefore, in contrast to aspirin, their antiplatelet actions are short-lived, generally no longer than 6 hours, depending on the half-life of the drug in the circulation.[84] Nonaspirin NSAIDs cause transient, dose-dependent, and only modest prolongations of the bleeding time, which usually do not even exceed the upper limit of normal. The effects of these drugs on clinical bleeding complications are less clear than those of aspirin. As recently reviewed,[84] conflicting results have been reported regarding effects of nonaspirin NSAIDs on perioperative blood loss.

A randomized, double-blind study found that indobufen, a reversible COX inhibitor, reduced the incidence of ischemic events in patients at risk for cardiogenic embolism.[85] Indobufen also has been demonstrated to be as effective as aspirin plus dipyridamole in preventing saphenous vein coronary artery bypass graft occlusion, with possibly fewer adverse events.[86]

Thromboxane Synthase Inhibitors and Thromboxane Receptor Antagonists

The lack of platelet specificity of COX inhibitors led to the development of thromboxane synthase (TxS) inhibitors. In addition to blocking TXA_2 synthesis, these drugs should have the advantage of diverting platelet-derived endoperoxides to endothelial cells to promote prostacyclin production (Figure 2). However, clinical experience with TxS inhibitors has been disappointing. Reversible TxS inhibitors have short plasma half-lives and suppress TXA_2 production incompletely.[87] Dazoxiben and other imidazole analogs have produced little or no clinical

benefit in Raynaud's phenomenon,[88] hepatorenal syndrome,[89] or coronary artery disease.[90]

Thromboxane synthase inhibitors may be relatively ineffective because endoperoxides, which accumulate with TxS inhibition, themselves exert platelet agonist actions by occupying and activating the same platelet TXA_2 receptors. Therefore, TXA_2/endoperoxide receptor antagonists represent a potentially more attractive antiplatelet approach.[91] Furthermore, dual TxS inhibitors and TXA_2/endoperoxide receptor antagonists should be able to simultaneously block TXA_2/endoperoxide action and augment platelet endoperoxide-derived prostacyclin synthesis. One such agent, ridogrel, has been found to have more potent antiplatelet effects than aspirin,[92] but was not superior to aspirin as an adjunct to thrombolytic therapy for myocardial infarction.[93]

Inhibitors of Platelet Aggregation: Platelet Membrane GpIIb/IIIa Antagonists

The actions of many of the antiplatelet agents reviewed above are restricted to single, specific platelet receptors or metabolic pathways. Global interruption of platelet function, which should improve clinical efficacy, may be achieved by blocking either the initial (adhesion) or final (aggregation) steps in the sequence of platelet activation.

Platelet aggregation induced by any stimulus is abolished if binding of fibrinogen (or vWf) to its GpIIb/IIIa receptors in inhibited. Two general strategies have been used to target this interaction: monoclonal antibodies to GpIIb/IIIa, and RGD analogs that compete with ligand binding to GpIIb/IIIa.[94,95] All of these agents are potent inhibitors of platelet aggregation and cause prolongation of the bleeding time.

The monoclonal antibody 7E3, developed by Coller,[96] has been extensively tested in animal and human antithrombotic trials. The immunogenicity of 7E3 can be circumvented by using "humanized" chimeric antibodies containing the variable regions of the mouse antibody joined to the constant regions of human antibody (c7E3, Abciximab). Monoclonal antibodies bind to and inhibit platelets irreversibly.

Peptides containing RGD ("disintegrins") have been isolated from snake venoms and leeches.[97] To reduce their antigenicity and improve metabolic stability and activity, a number of cyclic RGD peptides, peptidomimetic, and nonpeptide RGD mimetics have been synthesized.[94,95] Integrelin, based on a Lys-Gly-Asp sequence rather than RGD sequence, which may have improved specificity for GpIIb/IIIa receptors, has already undergone extensive clinical evaluation. Other RGD analogs, including more recently developed orally active agents, are in various phases of clinical testing.

The EPIC trial of c7E3, administered as a bolus and 12-hour infusion to more than 2,000 patients undergoing coronary angioplasty or atherectomy, found a 35% reduction in the composite-event rate, primarily in the rate of myocardial infarction and need for emergency revascularization.[98] Remarkably, this acute treatment also reduced the rate of clinically significant restenosis by 26% at 6 months of follow-up.[99] These results suggest that brief exposure to c7E3 at the time of initial revascularization converts a platelet-reactive vessel surface to a platelet-nonreactive one, a phenomenon referred to as blood-vessel passivation.[95] The optimal duration of GpIIb/IIIa blockade to achieve passivation has yet to be determined, and long-term therapy with orally active GpIIb/IIIa inhibitors may produce superior results. Initial experience with Integrelin in 150 patients undergoing coronary intervention demonstrated a trend toward reduction in acute ischemic end-point events at 30 days of follow-up.[100]

In unstable angina, Iamifiban, a nonpeptide GpIIb/IIIa antagonist, reduced the incidence of myocardial infarction and death,[101] confirming earlier pilot trials with Abciximab[102] and Integrelin.[103] The TAMI 8 pilot study reported improved coronary artery patency and a trend to fewer ischemic events with 7E3 (added to aspirin and heparin) following thrombolytic therapy for myocardial infarction.[104]

Increased bleeding complications, as noted in the EPIC study,[98] pose the major problem with all GpIIb/IIIa inhibitors, particularly when treatment is combined with invasive procedures; bleeding may be exacerbated by concomitant anticoagulation therapy. Future studies with these promising agents should determine the differences among the various GpIIb/IIIa inhibitors with regard to optimal routes of administration, pharmacokinetics, duration of effect, integrin affinity and specificity (including cross-reactivity with endothelial vitronectin receptors), antigenicity, and cost. Orally active drugs may provide highly effective long-term preventive therapy.

CONCLUSIONS

Enormous progress has been made in recent years in the development of clinically effective antiplatelet agents. The "holy grail" of antithrombotic therapy is the discovery of drugs that can discriminate between undesirable thrombosis and physiologically protective hemostasis, selectively inhibiting the former while preserving the latter to prevent bleeding complications. Since thrombosis has been viewed as hemostasis in the wrong place, the promise of an entirely effective and safe antiplatelet agent rests on further elucidating the molecular mechanisms of platelet activation.

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Clopidogrel – a Platelet Inhibitor which Inhibits Thrombogenesis in Non-Anticoagulated Human Blood Independently of the Blood Flow Conditions

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Summary

The goal of the present study was to investigate the effect of 7 and 14 days of daily oral administration of 75 mg clopidogrel on collagen-induced thrombogenesis in flowing non-anticoagulated human blood. Blood was drawn directly from an antecubital vein over immobilised collagen type III fibrils positioned in a parallel-plate perfusion chamber. The wall shear rates at the collagen surface were those characteristic for veins (100 s^{-1}), and for medium sized (650 s^{-1}) and moderately stenosed (2600 s^{-1}) arteries.

Clopidogrel ingestion reduced the thrombus volume significantly ($p < 0.05$) at 100 and 2600 s^{-1} (39 and 51% respectively). The β-thromboglobulin plasma levels were reduced concomitantly. However, it was not possible to measure accurately the thrombus volume at 650 s^{-1}, due to loose packing of the platelet thrombi. Transmission electron microscopy substantiated this observation and showed that clopidogrel profoundly reduced the platelet degranulation process ($p < 0.005$). The inhibitory effect of clopidogrel on platelet consumption by the growing thrombi resulted apparently in higher platelet concentration at the collagen surface, which enhanced the platelet-collagen adhesion at all three shear rates ($p < 0.05$). Despite the low deposition of fibrin on collagen, clopidogrel reduced significantly the fibrinopeptide A plasma levels and the fibrin deposition at shear rates below 650 s^{-1}. This was apparently a consequence of the reduced platelet recruitment and the lower activation of platelets, since activated platelets in thrombi promote deposition of fibrin.

Thus, it appears that clopidogrel inhibits thrombus formation in human blood primarily by interfering with platelet-platelet binding and the inhibition is effective independently of the wall shear rate.

Introduction

Platelets are prominent building blocks of arterial thrombi. An occluding platelet thrombus may prove fatal, depending on the location of the vessel involved. There exists a complex interplay between platelets, activated coagulation proteins, thrombus formation, and the development of atherosclerosis (1). Drugs which inhibit platelet function have proven valuable in diseases where platelets play a significant role in the pathophysiological processes. Numerous drugs interfering with various aspects of platelet functions have therefore been developed and tested in a variety of clinical trials.

Today aspirin is the most commonly used antiplatelet-drug. Aspirin has been shown to protect against primary and secondary myocardial infarction (2, 3), and to have a beneficial effect in patients with unstable angina (4–7). These disorders are together among the major precursors of death in the Western world.

Clopidogrel, a derivative of thieno-[2, 3-c]pyridine, is a new inhibitor of platelet aggregation and an antithrombotic agent. It is an ADP receptor blocker as suggested by various studies in man and animal (8–10).

The goal of the present study was to quantitate the antithrombotic effect of 7 and 14 days of clopidogrel administration on blood-collagen interactions in non-anticoagulated blood of healthy human volunteers at blood flow conditions characteristic of veins, healthy and stenosed arteries (wall shear rates of 100, 650 and 2600 s^{-1}, respectively). The human ex vivo model of thrombogenesis (11), which was used for this purpose, was previously validated in dogs as a model of thrombosis. It was found that the ex vivo thrombotic response correlated well with experimentally induced in vivo coronary thrombosis (12). Furthermore, data obtained with blood from patients suffering from various subtypes of von Willebrand disease and Haemophilia A correlates well with clinical findings in humans (13). Antiplatelet agents such as aspirin (14) and a thromboxane A_2 receptor antagonist (15) have also been evaluated with this technique, and of particular interest to the present study is that the antithrombotic effect of aspirin is limited to high wall-shear rates (14). This finding correlates well with the protective effect of aspirin on thrombotic events in patients with unstable angina (4–6).

Methods

Design of the Study

Eighteen healthy male subjects aged 19 to 37 years completed this open study. All volunteers were non-smokers and willing to abstain from alcoholic beverages during 24 h prior to the respective blood donations. All individuals were fully informed about the study, and had given in writing their free, informed consent for participation.

This work was performed at the Biotechnology Centre of Oslo, University of Oslo, Gaustadalléen 21, P. O. Box 1125, 0317 Oslo, Norway.

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Each volunteer was subjected to 14-day administration of clopidogrel (Sanofi Recherche, Toulouse, France). The drug was taken orally as a single daily dose of 75 mg (3 × 25 mg tablets). Safety measurements within 7 days before and 14 days after the medication (day 28) were used as individual controls for measurements after 7 and 14 successive days of active clopidogrel medication. Platelet adherence and thrombus formation on collagen type III fibrils were measured on the same four occasions using the ex vivo perfusion method (16) and the parallel-plate perfusion chambers developed by Sakariassen et al. (11, 17).

The protocol was approved by the appropriate ethics committee, and the study conducted according to the principles of the declaration of Helsinki.

Clinical and Laboratory Surveillance

General physical examination and laboratory safety investigation were performed in collaboration with Rikshospitalet, Oslo, before medication was started, at the end and 14 days after the end of medication. The laboratory safety data recorded were hemoglobin, hematocrit, total and differential white blood cell count, platelet count, liver function tests and urinalysis.

Platelet Aggregation

Eighteen ml of blood were drawn from an antecubital vein through a 19 gauge needle into 2 ml of 110 mM tri-sodium citrate solution. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by differential centrifugation. Platelet concentration in PRP was adjusted to 3×10^{11}/l by the addition of PPP. Platelet aggregation in PRP stirred at 1000 rpm at 37° C was induced by ADP (Sigma Chemical Co., St. Louis, Mo) at 1, 5 and 10 μM and by equine collagen (Collagen Reagent Horm, Munich, Germany) at 1 and 5 μg/ml, all final concentrations. The aggregation response was optically recorded for 4 min in an aggregometer device (Chrono-log model 440, Chrono-log Corp., Havertown, Pa).

Preparation of Collagen Surfaces

Type III collagen was purified to more than 95% homogeneity from a pepsin digest of human placenta by selective salt precipitation (18). A sample of the type III collagen was hydrolyzed in 6 M HCl in vacuum for 24 h, and the hydrolyzed material was analysed by a Biotronik LC 5000 amino acid analyser (Biotronik, Munich, Germany). The collagen concentration was estimated from the amino acid composition. Collagen fibrils were made by dialysis at 4° C against 20 mM Na_2HPO_4, pH 7.5 (19).

Washed Thermanox™ plastic cover slips (Miles Laboratories, Naperville, Il) were spray-coated with human placental type III fibrillar collagen using an air brush at a nitrogen operating pressure of 1 atm to a final density of about 20 μg/cm². The collagen coated cover slips were left for 16–20 hours at 22° C before they were used in the perfusion experiments (20). The fibrils do not trigger coagulation and the coating has been shown to give a maximal thrombogenic stimulus at 10 μg/cm² (final density on spray-coated cover slips). The thrombogenicity of these fibrils is much like that of intact artery subendothelium, although the latter surface induces much more fibrin deposition (21).

Ex Vivo Perfusions, Fixation and Embedding

Ex vivo perfusions (16) were performed at 37° C with parallel-plate perfusion chambers in which a collagen-coated cover slip was positioned (11, 17). After puncturing an antecubital vein with a no. 19 Butterfly Infusion Set (Abbott Lab, North Chicago, Il), blood was drawn directly over the collagen surface at a constant flow rate of 10 ml/min by an occlusive roller pump (LKB 2115 Multiperpex pump, Bromma, Sweden) placed distally to the chamber. The cross-sectional dimensions of the blood flow channel of the chamber, which determine the blood flow characteristics, were chosen to give wall shear rates characteristic of veins (100 s^{-1}), healthy medium sized arteries (650 s^{-1}) and mildly stenosed arteries (2600 s^{-1}).

The blood perfusions lasted for 5 min, and were immediately followed by a 20 s perfusion at 10 ml/min with a buffer containing 130 mM NaCl, 2 mM KCl, 12 mM $NaHCO_3$, 2.5 mM $CaCl_2$ and 0.9 mM $MgCl_2$ (pH 7.4) and a 40 s perfusion (10 ml/min) with fixation solution, consisting of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Post-fixation was performed with freshly prepared fixation solution at 4° C for 1 h. The cover slips were finally embedded in Epon (20).

Morphometry

Platelet-collagen and fibrin-collagen interactions were light microscopically quantified on semithin sections. The sections were cut perpendicular to the direction of the blood flow 1 mm downstream from the upstream edge of the cover slip. The sections were stained with basic fuchsin and toluidine blue (22).

Standard morphometry was used to assess the percentage of the surface covered by platelets (% platelet adhesion) and by fibrin (% fibrin deposition) (22), and the percentage of the adhered platelets covered with fibrin (11). The evaluations were carried out at 1000 × magnification using a Labphot-2 light microscope (type 104, Nikon, Japan).

Computer-assisted microscopic morphometry was used to quantify average thrombus area ($\mu m^2/\mu m$ sectional length). Thrombus volume ($\mu m^3/\mu m^2$) was derived from the sectional thrombus area as previously described (23). A Kontron Vidas image analysing unit (Zeiss, Eching/Munich, Germany) was used for the evaluations.

Transmission electron microscopy (Philips EM 301 Electron Microscope, Eindhoven, The Netherlands) was used to count the number of non-released secretory granules (α-granules and dense granules). At least two random sections were cut from the central part of the flow slit at the same axial position. Electron micrographs were then made of all thrombi/platelet aggregates visualised. The area of the thrombi was measured with a planimeter, a Kontron Vidas image analysing unit, directly on the electron micrographs. A ratio between the number of identified non-released secretory granules and the sectional area of the thrombi (arbitrary units) was calculated. A high ratio indicated less release of secretory granules and less degree of platelet activation. The evaluations were carried out on micrographs at a magnification of 21300×.

Fibrinopeptide A (FPA) and β-Thromboglobulin (β-TG)

FPA and β-TG levels in plasma were measured both proximal and distal to the perfusion chamber. After puncturing a vein at the start of a perfusion experiment, the first 5 ml of blood were collected into EDTA for determination of platelet count and hematocrit. The next 1.8 ml were collected into two Eppendorf tubes prefilled each with 0.1 ml anticoagulant mixture, consisting of 1000 U heparin + 1000 U aprotinin per ml of saline for FPA and by the method of Ludlam and Cash (24) for β-TG. The samples were kept on ice.

At 4 min perfusion time blood samples (2 × 0.9 ml) were collected immediately distal to the chamber. The tubing was punctured in a rubber-coated area positioned immediately distal to the chamber, between the perfusion chamber and the roller pump. The rubber coating of the silicon tubing prevented leakage of blood following the puncture. The blood sampling was performed manually and without switching off the pump. The blood samples were collected successively during 15 s, into 1 ml syringes prefilled with 0.1 ml of the FPA- or β-TG-anticoagulant mixture, and immediately chilled to 0° C. Further processing of the blood samples for quantification of the plasma levels of FPA and β-TG was according to the manufacturers of the respective kits, IMCO (Stockholm, Sweden) for FPA and Amersham (Amersham, UK) for β-TG.

Statistical Analysis

The changes from baseline (of natural logarithm transformed) FPA and β-TG values post-perfusion for each shear group and at each stage of the study were modelled by the one-way ANOVA; hypotheses of no change from baseline for each shear group were tested using the pooled error term. The significance of differences of group or paired data were calculated using Student's t-tests. P-values <0.05 were considered significant.

Results

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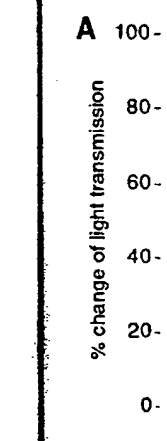

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Results

Clinical and Laboratory Data

Twenty-two volunteers were recruited for the study, and 18 completed according to the protocol. There were no serious adverse events related to the medication, and the volunteers who did not complete were withdrawn from the study for events unrelated to the study drug.

The clinical examination and laboratory safety data did not reveal significant clinical alterations in any parameter measured in the 18 donors completing the study. One volunteer contracted a severe viral infection at around the third sampling time and his results, although completed, are discounted from the analysis because of the possible confounding effects of the infection on both coagulation and platelet function.

Hematological Parameters

Hematocrit, white cell and platelet counts were measured in EDTA-blood at screening and before every perfusion and platelet aggregation experiment (Cell-Dyn™ 900 Hematology Analyzer, Sequoia-Turner Corp., Mountain View, Ca). An average drop in both hematocrit and platelet count of 4% was observed after 2 weeks of clopidogrel ingestion. Both reductions were probably due to hemodilution caused by the blood donations, since each volunteer donated approximately 400 ml

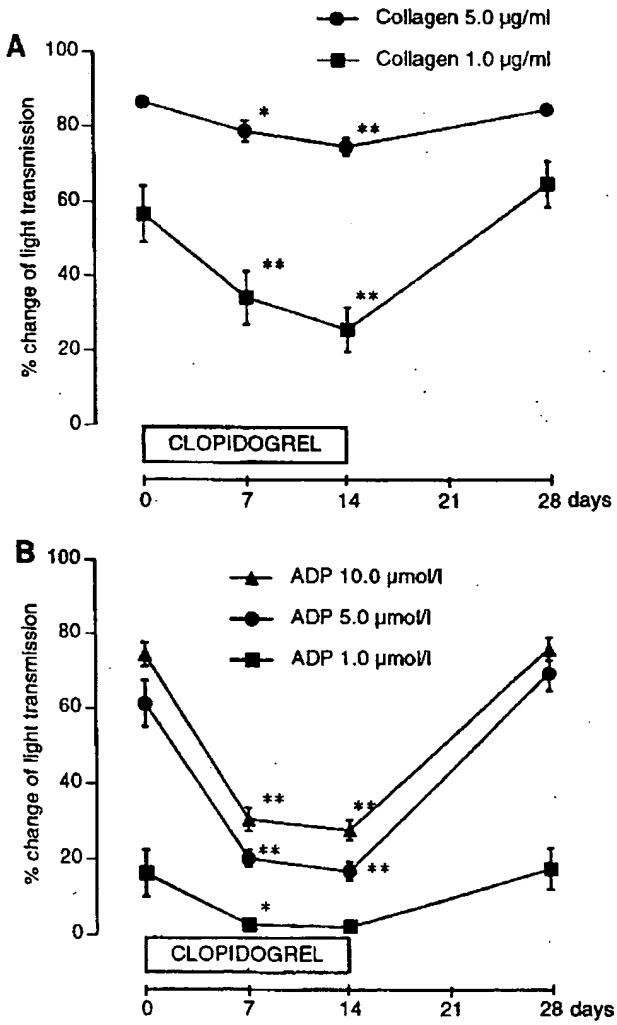

Fig. 1 Effect of clopidogrel on platelet aggregation induced by ADP and collagen in platelet-rich plasma (PRP). A. Collagen at final concentrations of 1 and 5 µg/ml. B. ADP at final concentrations of 1, 5 and 10 µM. Mean ± SEM (n = 18). *p <0.05 and **p <0.01 denote significant differences between platelet aggregation before and after clopidogrel ingestion (Student's t-test)

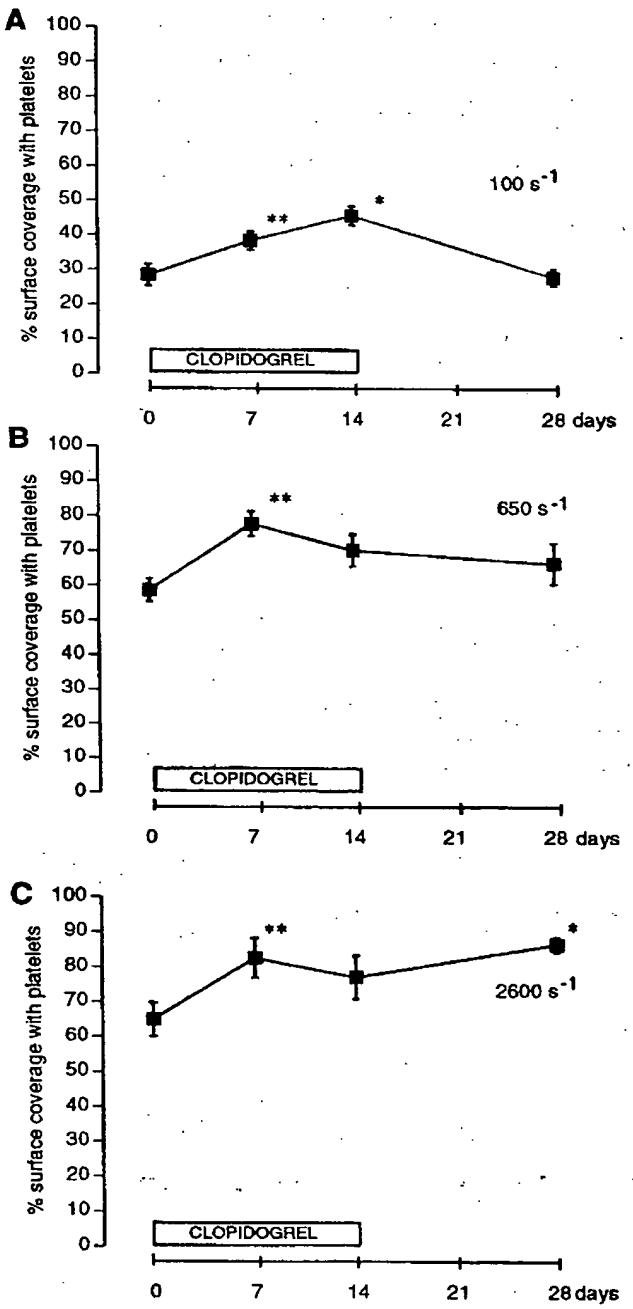

Fig. 2 Effect of clopidogrel on platelet adhesion (percent of surface covered by platelets) after 5 min perfusion at 10 ml/min in non-anticoagulated blood. Shear rates (A) 100 s^{-1}, (B) 650 s^{-1} and (c) 2600 s^{-1}. Mean ± SEM (n = 5–6). *p <0.05 and **p <0.01 denote significant differences between platelet adhesion before and after clopidogrel intake (Student's t-test)

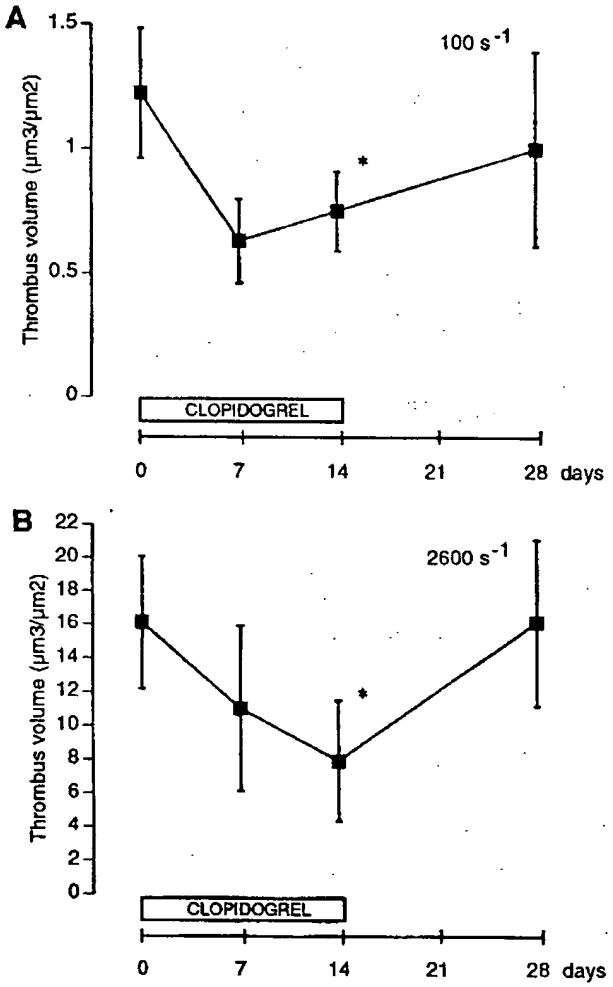

Fig. 3 Effect of clopidogrel on thrombus volume ($\mu m^3/\mu m^2$) after 5 min perfusion at 10 ml/min flow rate in non-anticoagulated blood. Shear rates (A) 100 s^{-1} and (B) 2600 s^{-1}. Mean SEM (n = 5–6). *p <0.05 denotes significant differences between thrombus volume before and after clopidogrel ingestion (Student's t-test)

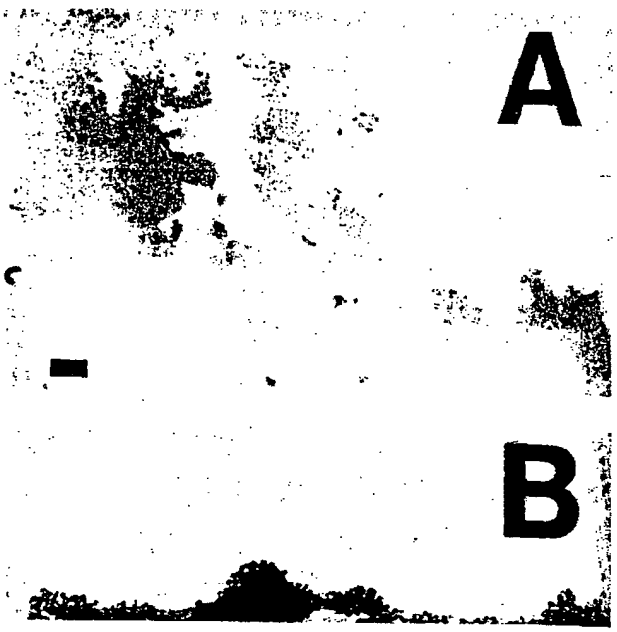

Fig. 4 Light micrographs of blood-collagen interactions after 5 min perfusion at shear rate 2600 s^{-1} (A) before and (B) after 2 weeks of clopidogrel ingestion. The collagen coat is not visible, since the staining procedure does not contrast the fibrils. Magnification 800×, bar represents 10 µm

blood. All individual hematocrits and platelet counts were within the normal range. A small non-significant drop in mean white cell count was observed after 2 weeks of clopidogrel.

Effect of Clopidogrel on Platelet Aggregation in Citrated PRP

Significant inhibition of ADP- and collagen-induced aggregation was observed both after one and two weeks of clopidogrel ingestion (Fig. 1). The inhibitory effect on platelet aggregation seemed generally stable between day 7 and day 14 of clopidogrel intake. Aggregation induced by 1 µg/ml equine collagen was inhibited by 46% ($p < 0.01$) after one week and by 61% ($p < 0.01$) after two weeks of clopidogrel ingestion. A concentration of 5 µg/ml collagen appeared to overcome the inhibition seen at the lower concentration, although a small inhibition remained statistically significant (9 and 14%; $p < 0.01$).

Aggregation induced by 1.0 µM ADP during the control periods was limited with large intersubject variability. This limited aggregation response was effectively abolished by clopidogrel treatment. With 5.0 and 10.0 µM/l ADP, strong aggregations were obtained in the control periods, which were markedly (59–73%, $p < 0.01$) inhibited by clopidogrel ingestion.

There were no significant differences between the groups of volunteers assigned to each perfusion group concerning baseline platelet aggregation or response to clopidogrel.

Effect of Clopidogrel on Platelet-Collagen Adhesion

Clopidogrel-ingestion significantly increased platelet-collagen adhesion at each wall shear rate both after one and two weeks of clopidogrel administration (Fig. 2).

The increase in platelet adhesion at the wall shear rate of 100 s^{-1} after one week of clopidogrel ingestion was 35% ($p < 0.01$), and after two weeks 50% ($p < 0.05$). At 650 s^{-1} the corresponding increase was 33% ($p < 0.01$) and 20% (non-significant). At 2600 s^{-1} the increase in platelet-collagen adhesion after one week of clopidogrel ingestion was 27% ($p < 0.01$), and by two weeks 19% (non-significant). The increase in platelet-collagen adhesion at 2600 s^{-1} persisted even at day 28 (33%; $p < 0.05$).

Effect of Clopidogrel on Thrombus Volume

Significant reductions of thrombus volume after clopidogrel ingestion were observed at both 100 s^{-1} and at 2600 s^{-1} (Fig. 3). The thrombus volume at 100 s^{-1} was decreased by 48% after one week of clopidogrel ingestion, and by 39% ($p < 0.05$) after two weeks. At 2600 s^{-1}, the corresponding decrease was 32% (non-significant) and 51% ($p < 0.05$) (Fig. 3). Representative light micrographs of thrombi formed at 2600 s^{-1} before and after two weeks of clopidogrel ingestion are shown in Fig. 4.

At a wall shear rate of 650 s^{-1} we encountered a problem in the use of computer-assisted morphometry. While the platelet thrombi before

Fig. 5 Transmission electron microscopy of thrombi forming on collagen after 5 min perfusion at shear rate 650 s^{-1} (A) before and (B) after 2 weeks of clopidogrel ingestion. Note the dense appearance of the thrombus before clopidogrel ingestion, in contrast to the loosely packed thrombus with swollen non-degranulated platelets following clopidogrel intake. Magnification 5700×

and 14 days after ingestion of clopidogrel seemed dense and rather homogenous, the thrombi during clopidogrel ingestion appeared morphologically different, consisting of platelets very loosely attached to one another. The borders of the thrombi were impossible to assess properly with computer-assisted morphometry. Electron microscopy was therefore performed. Sections from before drug intake were compared to those after 14 days of clopidogrel intake at the wall shear rate of 650 s^{-1}. In all volunteers the ratio of non-released secretory granules to sectional thrombus area was significantly increased, indicating that clopidogrel decreased the platelet release reaction/activation. The ratio (arbitrary units) increased from 4.7 ± 6.3 to 44.8 ± 14.8 (mean ± SD, $p < 0.005$). Representative electron micrographs show a loosely packed platelet thrombus with swollen non-degranulated platelets following clopidogrel ingestion, in contrast to densely packed and partly fused platelets before clopidogrel intake (Fig. 5).

Effect of Clopidogrel on Platelet Activation

Pre-perfusion β-TG plasma levels were relatively constant through the study and between shear groups (pooled geometric mean 31.7 ng/ml, range 13–400 ng/ml). Post-chamber levels were on average 4–10 times higher at the baseline measurement. Reductions of the post-chamber β-TG plasma concentrations were observed at all shear rates following clopidogrel ingestion (Fig. 6). At 100 and 650 s^{-1}, β-TG concentrations were changed by perfusion after one and two weeks of treatment and the differences in postperfusion levels compared to baseline were statistically significant ($p < 0.05$). At 2600 s^{-1}, β-TG levels were increased by perfusion, but although the level generated was less than at baseline the difference was not statistically significant. Two weeks after the end of clopidogrel treatment the postperfusion concentrations of β-TG had returned towards baseline levels for all shear groups.

Effect of Clopidogrel on Fibrin Deposition

Fibrin deposition (percent of surface covered with fibrin) on collagen before clopidogrel ingestion was low. Clopidogrel-induced

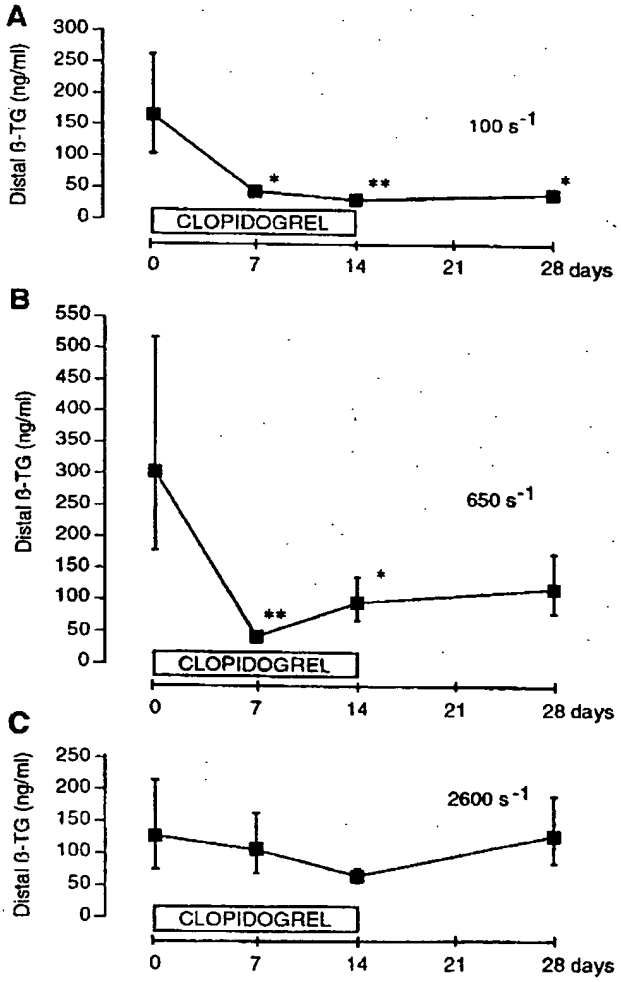

Fig. 6 Effect of clopidogrel on post-chamber β-TG plasma levels at 4 min during a 5 min perfusion at 10 ml/min in non-anticoagulated blood. Shear rates (A) 100 s^{-1}, (B) 650 s^{-1} and (C) 2600 s^{-1}. Geometric mean ± SEM (n = 5–6). *$p < 0.05$ and **$p < 0.01$ denote significant differences between β-TG values before and after clopidogrel ingestion (Student's t-test). Note that the area of the collagen surface exposed to blood at 2600 s^{-1} is 62.5% of the area at 100 s^{-1} and 650 s^{-1}

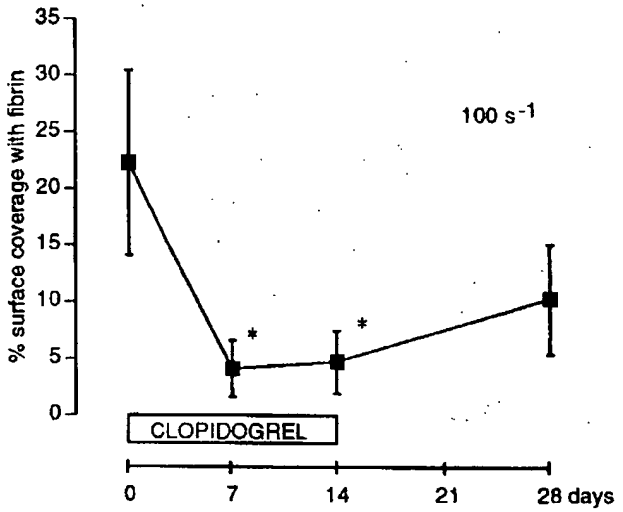

Fig. 7 Effect of clopidogrel on fibrin deposition (percent of surface covered by fibrin) after 5 min perfusion at 10 ml/min in non-anticoagulated blood. Shear rate 100 s^{-1}. Mean ± SEM (n = 6). *p <0.05 denotes significant differences between fibrin deposition before and after clopidogrel ingestion (Student's t-test)

reduction of fibrin deposition was significant ($p < 0.05$) at 100 s^{-1} (Fig. 7). A parallel drop was observed in the percentage of the platelets covered with fibrin, from 31.0 ± 6.4% before clopidogrel to 3.8 ± 3.1% after 14 days of clopidogrel ingestion (mean ± SEM, $p < 0.005$). At 650 s^{-1} and 2600 s^{-1} the surface coverage with fibrin was less than 2% both before and after clopidogrel ingestion, thus virtually absent.

Effect of Clopidogrel on FPA Plasma Levels

Pre-perfusion FPA plasma levels were rather constant through the study and between the shear groups (pooled geometric mean 1.65 ng/ml, range 0.8–21.1 ng/ml). Post-chamber values were on average 5 to 50 times higher at baseline. The small increase in post-perfusion FPA plasma levels at 2600 s^{-1} was little affected by clopidogrel treatment (Fig. 8). However, the much larger increase at 100 and 650 s^{-1} was reduced during clopidogrel treatment. The FPA generation was significantly lowered at 100 s^{-1} ($p < 0.05$), thus paralleling the drop in fibrin deposition on platelets and collagen (Fig. 7).

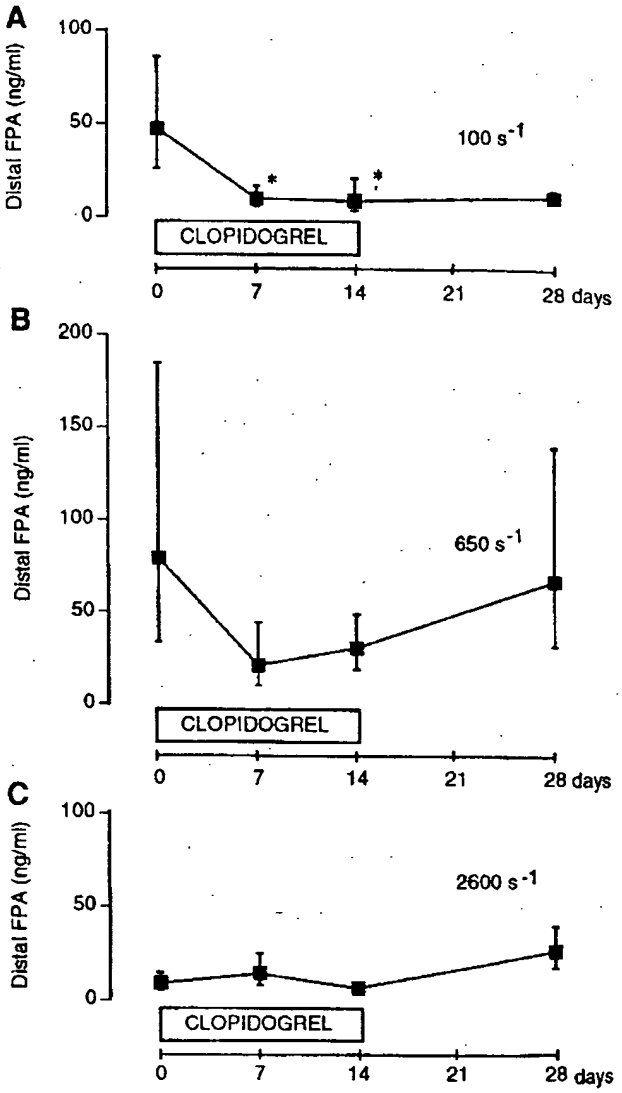

Fig. 8 Effect of clopidogrel on post-chamber FPA plasma levels at 4 min during a 5 min perfusion at 10 ml/min in non-anticoagulated blood. Shear rates (A) 100 s^{-1}, (B) 650 s^{-1} and (c) 2600 s^{-1}. Geometric mean ± SEM (n = 5–6). *p <0.05 denotes significant differences between FPA plasma values before and after clopidogrel ingestion (Student's t-test). Note that the area of the collagen surface exposed to blood at 2600 s^{-1} is 62.5% of the area at 100 s^{-1} and 650 s^{-1}

Discussion

Clopidogrel is a newly developed anti-platelet drug chemically a derivative of thienopyridine. The present study was undertaken to quantify the effect of 7 and 14 days of clopidogrel administration on thrombus formation in flowing non-anticoagulated blood of healthy volunteers in a human ex vivo model of thrombogenesis (11). The blood flow conditions were characterized by wall shear rates encountered in veins (100 s^{-1}), medium sized arteries (650 s^{-1}) and moderately stenosed arteries (2600 s^{-1}).

Clopidogrel reduced the thrombus volume significantly both at 100 s^{-1} and at 2600 s^{-1}. Two weeks of daily oral administration of 75 mg reduced the average thrombus volume by 51% at the highest arterial wall shear rate and by 39% at the venous shear rate. The post-chamber β-TG plasma levels and platelet aggregation in PRP induced by either ADP or collagen were concomitantly reduced. It appears that clopidogrel is more effective than aspirin, since aspirin has no significant anti-thrombotic effect at low arterial wall shear rates in the same model (15).

A consequence of the clopidogrel-induced reduction of thrombus volume at 100 and 2600 s^{-1} was the concomitantly enhanced platelet adhesion. Lower consumption of platelets by smaller thrombi after ingestion of a platelet inhibitor increases the platelet concentration in the blood layers streaming adjacent to the collagen surface (21). Thus, more platelets are available to adhere to the collagen. Increased platelet adhesion was observed at all shear rates following one and two weeks of clopidogrel administration, which is in accordance with a reduced incorporation of platelets into the thrombi. This enhancement also indicates that clopidogrel has no effect on the platelet-collagen adhesion. Thus, platelet-collagen adhesion seems not to be affected by ADP, unlike collagen-induced platelet aggregation.

The effect of clopidogrel on thrombus formation at 650 s^{-1} was morphologically different from that observed at 100 and 2600 s^{-1}. These thrombi appeared as loosely packed platelet masses with swollen and non-degranulated platelets. The morphological appearance of the predominantly non-degranulated platelets coincided with the significant reduction of the post-chamber plasma levels of β-TG and the enhanced platelet-collagen adhesion. Thus, these findings support an anti-thrombotic effect of clopidogrel also at this shear condition, even though it was impossible to verify this by computer-assisted morphometry at the light microscopical level.

The partial interruption of the thrombotic response by clopidogrel has been ascribed to interference of the drug with the platelet ADP receptor mediating the inhibition of adenylate cyclase (8–10). From this study it is apparent that clopidogrel interferes with the platelet-platelet binding and not with the platelet-collagen adhesion. The kinetics of the apparent shear rate independent interruption of the platelet-platelet binding induced by clopidogrel was previously observed in Glanzmann's thrombasthenia (25), a bleeding disorder caused by deficiency of the platelet membrane glycoprotein IIb–IIIa complex (26). However, the reduction in thombus formation induced by the dose of clopidogrel used in this study was less than seen in blood from patients with Glanzmann's thrombasthenia.

Reduction of fibrin-collagen deposition, fibrin deposition on platelets and post-chamber FPA plasma levels following clopidogrel ingestion was apparently caused by the hampered platelet-platelet interaction. It has previously been demonstrated that activated clotting factors bind to the plasma membrane of platelets and leukocytes (11, 27–29), resulting in pronounced amplification of the clotting process (20, 31). Furthermore, time-course studies with the collagen surface used in this investigation have shown that fibrin deposition only occurs subsequent to significant platelet-thrombus formation (27). Thus, the apparent anticoagulant effect of clopidogrel seems rather to be a consequence of the altered platelet response at the thrombogenic surface.

Fourteen days after ending clopidogrel ingestion, the surface coverage of platelets at 2600 s^{-1} and the distal β-TG levels at 100 s^{-1} were still significantly different from the control values. However, neither platelet aggregation nor thrombus volume point to any persisting major activity of clopidogrel on thrombogenesis.

Our data is consistent with the view that the shear rate independent anti-thrombotic effect of clopidogrel is due to interference with the platelet-platelet interaction. This interference hampers the thrombotic response and indirectly the coagulant events at the reactive surface. It is also apparent that clopidogrel has a larger anti-thrombotic potential than aspirin, at least in the ex vivo thrombosis model. The collagen-induced thrombus formation is interrupted irrespective the laminar blood flow conditions. However, the collagen surface employed is non-procoagulant (11), while situations with atherosclerotic plaque rupture also may include exposure of tissue factor to blood (32, 33). The effect of clopidogrel on thrombus formation when a procoagulant surface is exposed to the blood stream at various wall shear rates remains to be established.

Acknowledgements

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$P2Y_{12}$, A New Platelet ADP Receptor, Target of Clopidogrel

Jean-Marc Herbert, Ph.D.[1] and Pierre Savi, Ph.D. [1]

ABSTRACT

Clopidogrel is a potent antithrombotic drug that inhibits ADP-induced platelet aggregation. The results of large clinical trials have demonstrated an overall benefit of clopidogrel over aspirin in the prevention of vascular ischemic events (myocardial infarction, stroke, vascular death) in patients with a history of symptomatic atherosclerotic disease. The antiaggregating effect of clopidogrel is attributed to an irreversible inhibition of ADP binding to a purinergic receptor present at the platelet surface. Clopidogrel is not active in vitro and can be considered a precursor of an active metabolite formed in the liver. The chemical structure of this active metabolite and its biological activity have been described recently. Several purinergic receptors have been described on platelets; $P2X_1$, a calcium channel, and $P2Y_1$ a Gq-coupled seven-transmembrane domain receptor, have been found not to be antagonized by clopidogrel. Another G_i-coupled receptor (named $P2Y_{12}$) has been recently cloned and stably expressed in CHO cells. These cells displayed a strong affinity for ^{33}P-2MeS-ADP, a stable analogue of ADP, the binding characteristics of which corresponded in all points to those observed on platelets. The binding of ^{33}P-2MeS-ADP to these cells was strongly inhibited by the active metabolite of clopidogrel with a potency that was consistent with that observed for this compound on platelets. In these transfected CHO cells, as in platelets, ADP and 2MeS-ADP induced adenylyl cyclase downregulation, an effect that was inhibited by the active metabolite of clopidogrel. These results demonstrate that this receptor corresponds to the previously called "P2t" platelet receptor and show that the active metabolite of clopidogrel binds in a covalent manner to this receptor, thus explaining how it blocks the aggregating effect of ADP on platelets.

KEYWORDS: $P2Y_{12}$, ticlopidine, clopidogrel

Educational Objectives: Upon completion of this article, the reader should be able to (1) understand the mechanism of action of clopidogrel on platelets; (2) understand the role of $P2Y_{12}$ in platelet aggregation; (3) appreciate the interest of $P2Y_{12}$ blocking for the secondary prevention of thrombosis in ateroclerotic patients.

Over the last decade, considerable interest has been focused on the role of platelets and platelet inhibitor therapy in atherosclerosis-derived diseases. The well-established role of platelets in arterial thrombosis has provided the rationale for many drugs that inhibit platelet functions.[1] The treatment of cardiovascular diseases and, in particular, ischemic heart disease has been unquestionably transformed by the use of antiplatelet

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therapy. Fortunately, there has been remarkable growth in our understanding of the molecular mechanisms of platelet aggregation, and several new antiplatelet agents have recently emerged.

Ticlopidine was discovered in 1972 and developed as an antithrombotic drug some years later. In 1978, ticlopidine was introduced on the market with a very narrow therapeutic indication: the extracorporeal circulation. Then, taking advantage of its clinical benefits, it became a useful antithrombotic drug. Ticlopidine has been shown to exhibit beneficial effects in patients with transient ischemic attack, reversible ischemic neurological deficit, or minor stroke[2]; in patients with a recent history of major ischemic cerebral event related to atherosclerosis[3]; and in patients with peripheral vascular disease.[4] In this high-risk population, ticlopidine reduced cardiovascular morbidity and mortality by 60%, whereas no evidence of aspirin efficacy was shown.

Clopidogrel was discovered in 1986 and approved in occidental countries in 1997. This compound has been demonstrated to prevent cardiovascular death in atherosclerotic patients in CAPRIE, a randomized phase III, triple-blinded clinical trial enrolling more than 19,000 patients with atherosclerotic disease. In this trial, it was shown that clopidogrel was more efficacious than aspirin in reducing the combined risk of ischemic stroke, myocardial infarction, or vascular death, and it was suggested that it might be used for widespread prevention of fatal or nonfatal ischemic systemic events.[5] The CAPRIE study also showed that the overall safety profile of clopidogrel was at least as good as that of medium-dose aspirin.

More recently, the results of the Clopidogrel in Unstable Angina to Prevent Recurrent Ischemic Events (CURE) study have been published.[6] This study is the first trial to investigate the potential role of clopidogrel in the treatment of patients with unstable angina (UA) and non-Q-wave myocardial infarction (NQMI) in the emergency setting and at long term. In this study, 12,562 patients across 482 centers in 28 countries admitted into hospital with suspected acute coronary syndrome (ACS) were recruited. Randomization of patients occurred within 24 hours of symptom onset. Qualifying patients were treated with a loading dose of clopidogrel 300 mg orally or with matching placebo on the first day. Acetylsalicylic acid (ASA, or aspirin) 75 to 325 mg daily was started simultaneously with the blinded study drug (or continued if patients were already taking ASA). Clopidogrel (75 mg once daily) or matching placebo was continued for 3 months to 1 year with additional follow-up visits at 6, 9, and 12 months. All patients recruited to CURE received ASA as part of standard therapy. Standard therapy could include other cardiovascular agents such as heparin/low-molecular-weight heparin, GPIIb/IIIa inhibitors, beta-blockers, statins, ACE inhibitors, calcium channel blockers, or coronary intervention at the physician's discretion. The CURE results show a highly significant and clinically important benefit for clopidogrel on top of standard therapy (including ASA) with a 20% relative risk reduction in the primary endpoint ($p < 0.001$; Table 1). The Kaplan-Meier curves began to separate as early as 2 hours after the clopidogrel loading dose. The curves continued to separate throughout the course of the trial, confirming short- and long-term benefits for the use of clopidogrel on top of standard therapy (including ASA). As expected, because both clopidogrel and ASA are platelet inhibitors, the main side effect was bleeding. There was an increase of major bleeds in the clopidogrel and ASA group of 1% (2.7 to 3.7%, $p = 0.001$) compared with placebo and ASA. Life-threatening bleeds were not significantly increased (1.8 to 2.2%). Minor bleeds increased from 2.4 to 5.1% ($p < 0.001$), respectively (Table 2). Therefore, CURE, the largest trial conducted to date in patients with UA and NQMI, demonstrates that clopidogrel on top of standard therapy (including ASA) is highly beneficial in patients with UA or NQMI, both early on (within hours of onset of symptoms) and in the long-term situation.

PHARMACOLOGY

Ticlopidine and clopidogrel belong to the thienopyridine family of drugs and present only minor structural differences. However, the presence of a methoxy carbonyl group on the benzylic position in the clopidogrel

Table 1 CURE Trial: Efficacy Results

Primary Endpoint	Clopidogrel on Top of Standard Therapy* ($n = 6259$)	Standard Therapy* ($n = 6303$)	Relative Risk	p value
CV death, MI, stroke (%)	9.3	11.4	0.80	0.00009
CV death (%)	5.1	5.5	0.93	
MI (%)	5.2	6.7	0.77	
Stroke (%)	1.2	1.4	0.86	
Non-CV death (%)	0.7	0.7	0.91	

*Including ASA.

Table 2 CURE Trial: Bleeding Results

Endpoint	Clopidogrel on Top of Standard Therapy, Including ASA (%, $n = 6259$)	Standard Therapy, Including ASA (%, $n = 6303$)	Relative Risk	p value
Major bleeding	3.7	2.7	1.38	0.001
Life-threatening bleeding	2.2	1.8	1.21	0.13
Other major (non-life-threatening) bleeding	1.5	0.9	1.70	0.002
Minor bleeding	5.1	2.4	2.12	<0.001
Transfusions of >2 units of blood	2.8	2.2	1.30	0.02

molecule provides an increased pharmacological activity and a better safety profile to this drug. Clopidogrel is an S enantiomer and, when tested in animals, the corresponding R enantiomer was devoid of antithrombotic activity, indicating that this position is crucial for the pharmacological activity of the drug. The antithrombotic activity of thienopyridines has been demonstrated in several animal species and models of arterial thrombosis, with some of them being insensitive to aspirin.[7,8] In various arterial-type models of thrombosis, clopidogrel exhibited a potent, dose-dependent antithrombotic activity, being approximately 50 times more potent than ticlopidine and about 100-fold as active as aspirin.[7]

In experiments aimed at determining the role of platelets in experimental venous thrombosis, we showed that ADP-mediated platelet activation played a major role in the development of venous thrombosis under low thrombogenic conditions[9] and suggested that clopidogrel may be of therapeutic interest in pathologies involving venous thrombosis. A recent study from our laboratory[10] gave further insight into these processes, showing that clopidogrel was able to alter thrombin generation in rat platelet-rich plasma and to decrease the platelet-induced procoagulant activity of endothelial cells,[11] therefore confirming that ADP-induced platelet aggregation is of particular importance in the triggering of venous-type thrombosis.

Because these compounds have been chosen for their ability to affect platelet aggregation when tested ex vivo, their antithrombotic activity has been attributed to this property, but some attempts have also been made to find other pharmacological effects of the thienopyridines that could be relevant for their antithrombotic effects. They include decrease of circulating fibrinogen levels,[12] increase of erythrocyte filterability,[13] or stimulation of nitric oxide production.[14] However, these effects, although contributory to the overall protective effect of these drugs, seem to be of secondary importance with regard to their antiplatelet activity.

The effects of ticlopidine and clopidogrel on platelet function have been extensively studied. A reduction of platelet aggregability has been reported for numerous agonists, but the effects against ADP were the most frequently observed.[7] Antagonism of the fibrinogen receptor Gp IIb-IIIa,[15] inhibition of prostanoid generation,[16] activation of adenylyl cyclase, or inhibition of phosphodiesterases[17] were first proposed as mechanisms responsible for the antiaggregating activity of these compounds, but these hypotheses were invalidated in subsequent studies,[18-21] and it is now accepted that these compounds are selective inhibitors of ADP-induced aggregation.[22]

METABOLISM

Ticlopidine and clopidogrel need to be administered in vivo to exhibit an antiaggregating activity. However, some direct effects of thienopyridines in vitro have been reported: inhibition of platelet aggregation,[23] inhibition of mitochondrial oxidative metabolism,[24] and anti-angiogenic[25] and pro-apoptotic effects.[26] Nevertheless, these effects (most of them being observed at nonrelevant doses) do not seem to account for the ex vivo antiaggregant activity of these drugs, responsible for their antithrombotic properties.

The antiaggregating activity of ticlopidine only occurs after repeated oral administrations,[27] whereas a similar effect is obtained approximately 2 hours after an oral or intravenous administration of a single dose of clopidogrel.[7] The achievement of an antiaggregating effect only after an in vivo administration suggests that the thienopyridines do not act directly on platelets and indicates that they must produce an antiaggregant active substance through a metabolic process. A study performed on clopidogrel confirmed this hypothesis.[28] In this study, we showed that the liver was the metabolic site from which the antiaggregant activity of clopidogrel originates. This was demonstrated by functional hepatectomy achieved by inserting a portal-jugular shunt that abolished the antiaggregant effect of clopidogrel and also by means of a perfused liver. We further showed that the hepatic bioactivation of clopidogrel required a cytochrome P450-1A-dependent metabolism.[29]

A study of the metabolism of ticlopidine resulted in the identification of about 20 separate metabolites,[8] representing approximately 30% of the initial compound, but none of them had any significant in vitro activity. The other metabolites representing approximately 70% of the initial compound have not yet been identified, but no study has been able to demonstrate an antiaggregant activity in the plasma of treated subjects.[30] This suggests that the active metabolite(s) circulates at very low concentrations and/or may have a very short half-life. Furthermore, because the platelets of clopidogrel-treated subjects remain resistant to ADP even after washing, the antiplatelet effects of clopidogrel are irreversible.[30] The inhibition of platelet aggregation continues after the end of treatment, and the rate at which aggregation is restored correlates closely with platelet production.[7,8]

These observations therefore suggested the presence of an active metabolite of clopidogrel, produced by the liver, acting in an irreversible manner on platelets. This compound has been recently purified and its chemical structure determined.[31] In this study, the active metabolite of clopidogrel has been prepared by incubating 2-oxo-clopidogrel with human liver microsomes. 2-Oxo-clopidogrel was used instead of clopidogrel because it not only has been shown to be generated from clopidogrel by the liver in a cytochrome P450-dependent manner[29,32] and to show a higher antiaggregant activity ex vivo but it also enabled us to generate sufficient amounts of the active metabolite of clopidogrel to determine its chemical structure and biological activity. The active metabolite of clopidogrel noncompetitively antagonized the binding of ^{33}P-2MeS-ADP, a stable analogue of ADP, on platelets and selectively inhibited ADP-induced aggregation, with no effects on ADP-induced shape change and epinephrine-induced aggregation, all activities that resembled the effects observed ex vivo after the administration of clopidogrel. Adenylyl cyclase downregulation was also inhibited by the active metabolite, when induced by ADP, whereas epinephrine-induced downregulation of adenylyl cyclase was not affected, as already described with clopidogrel.[21] All these effects were not observed with the corresponding metabolite from SR25989, the inactive enantiomer of clopidogrel. These effects were obtained after a short incubation period (1 hour, 20°C) under experimental conditions where an effect of the parent compound has never been found. From our previous[31,32] and present experiments, a possible metabolic pathway leading to the formation of this active metabolite was tentatively deduced (Fig. 1). In the liver, clopidogrel is metabolized into 2-oxo-clopidogrel through a cytochrome P450-dependent pathway. This intermediate is then hydrolyzed and generates the highly labile active metabolite that reacts as a thiol reagent, with the ADP receptors on platelets, when crossing the liver. This in situ effect could account for the absence of an antiaggregating activity in the plasma.[30] That ADP acted as a competitor to the irreversible inhibition of the ^{33}P-2MeS-ADP binding by the active metabolite of clopidogrel strongly supports our hypothesis that the ADP receptor is the target of the active metabolite. The lack of effect of the metabolite(R) clearly demonstrated that the interaction of the active metabolite was also highly dependent on its stereoisomery. Clopidogrel and ticlopidine are thought to have similar pharmacological effects. Both inhibit the binding of 2MeS-ADP,[33] ADP-induced downregulation of the adenylyl cyclase in platelets,[21] and ADP-induced platelet aggregation.[7,34] All these data indicate that the $P2Y_{12}$ (formerly P2t) receptor can be considered the main target of these thienopyridines. However, because the active metabolite of clopidogrel cannot be generated from ticlopidine, it should act via two different active metabolites. Indeed, the active metabolite of clopidogrel contains a methylester function on carbon 10. This function is present on clopidogrel but does not exist on ticlopidine. Therefore, because such an alkylation process cannot occur in vivo,

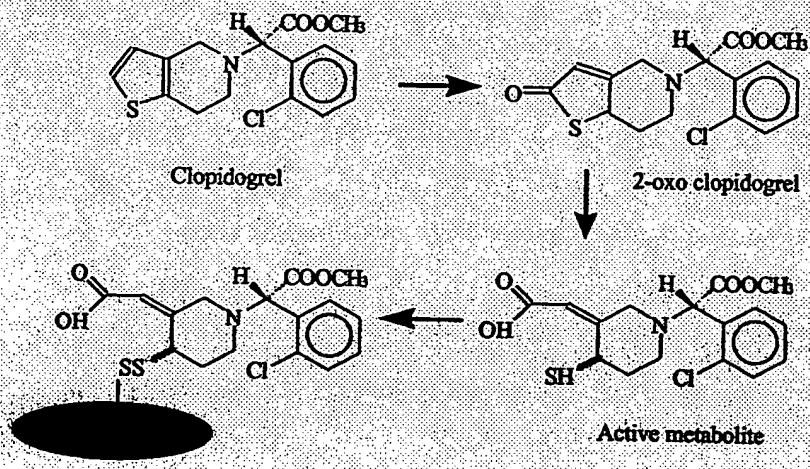

Figure 1 Scheme for the mechanism of clopidogrel action on platelets. Clopidogrel is converted into 2-oxo-clopidogrel by P450 monooxygenase-dependent metabolism. Hydrolysis of 2-oxo-clopidogrel generates the active metabolite. This compound reacts as a thioreactive reagent with the thiol of an amino acid (putatively a cysteine) of the platelet P2tAC receptor.

one can assume that the active metabolite generated from ticlopidine is different from that described here for clopidogrel.

MODE OF ACTION OF THIENOPYRIDINES

Effect on Platelet ADP Receptors

As a result of the specificity of thienopyridines for the effects of ADP, their effect on the platelet ADP receptor has been investigated. In healthy volunteers, Mills et al.[35] first demonstrated that clopidogrel inhibited the binding of ^{32}P-2-MeS-ADP to platelets. A similar study performed by us in the rat with ^{3}H-2-MeS-ADP confirmed these results.[33] In this study, we showed that treatment with clopidogrel reduced the ligand-binding capacity without modifying its affinity (Fig. 2). The reduction in the number of platelet ADP-binding sites correlated with the dose of clopidogrel administered as well as with inhibition of aggregation and suppression of the inhibition of adenylyl cyclase induced by ADP and 2-MeS-ADP. Similar results were obtained after treatment with ticlopidine.[33] Moreover, two cases of inherited disorders, mimicking the effects of these drugs, have been described.[36,37] For both patients, a strong decrease in ADP or 2-MeS-ADP binding on platelets has been observed.

A detailed study of the effect of clopidogrel on the binding of ^{3}H-2-MeS-ADP has demonstrated that there were two distinct populations of sites on the platelet surface.[38] One population of high-affinity sites might be responsible for the ADP-induced shape change. These receptors were not antagonized by high doses of clopidogrel, and this was also the case for the shape change that persisted even after the administration of a 10-fold excess of the optimal antiaggregating doses of clopidogrel. The low-affinity binding sites were specifically antagonized by a treatment with thienopyridines and, as a result of this antagonism, ADP-induced platelet aggregation and inhibition of adenylyl cyclase occurred. This clearly demonstrates that the low-affinity ADP receptors mediate events essential in the aggregation process.[38]

It is now well established that platelet aggregation by ADP requires the activation of two distinct receptors[39]: the $P2Y_1$ receptor, coupled to phospholipase C via G_q, and the $P2Y_{12}$, a recently characterized purinoreceptor coupled to G_{i2} and associated with adenylyl cyclase inhibition. As described earlier, the $P2Y_1$ receptor is the high-affinity receptor, responsible for the initial platelet shape change, whereas the $P2Y_{12}$ receptor (low-affinity receptor) plays a role in the amplification of platelet aggregation and thrombus stabilization (Fig. 3). However, the proaggregatory role of the $P2Y_{12}$ receptor is definitely not a consequence of adenylyl cyclase inhibition but probably involves the activation of kinases downstream of G_i.[40]

Thienopyridine-Resistant Platelet Purinoceptors: P2Y1

The clopidogrel-resistant effects of ADP were attributed to the platelet high-affinity receptors, which have been recently identified as $P2Y_1$.[41] This receptor represents almost 20% of the receptors recognized by 2-MeS-ADP at the platelet surface. When expressed in Jurkat cells, $P2Y_1$ behaves as a Gq-coupled receptor triggering the activation of inositol phosphate metabolism and cytosolic calcium increase. These effects are inhibited by A3P5P.[42] As in these cells, stimulation of platelets with low ADP concentrations induces shape

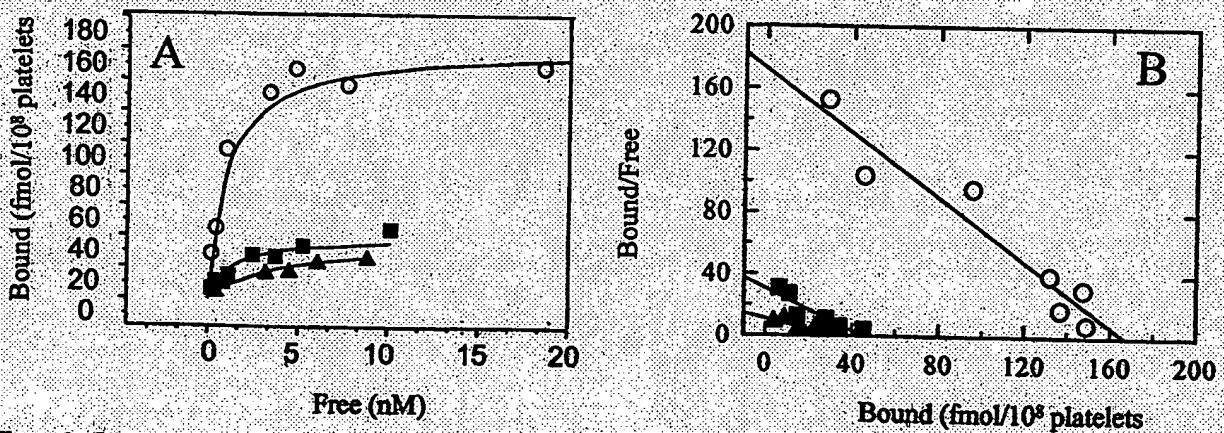

Figure 2 Effect of clopidogrel on the binding of ^{3}H-2-MeS-ADP to rat platelets ex vivo. (A) Platelets isolated from rats treated with clopidogrel (10 mg/kg, σ), ticlopidine (200 mg/kg.day, 3 days, ▪), or the vehicle (μ) were incubated for 15 minutes at 37°C with increasing concentrations of ^{3}H-2MeS-ADP. Specific binding is given by the difference between total and nonspecific binding determined in the presence of 1 mM ADP. (B) Scatchard plots of the specific binding of ^{3}H-2-MeS-ADP to rat platelets.

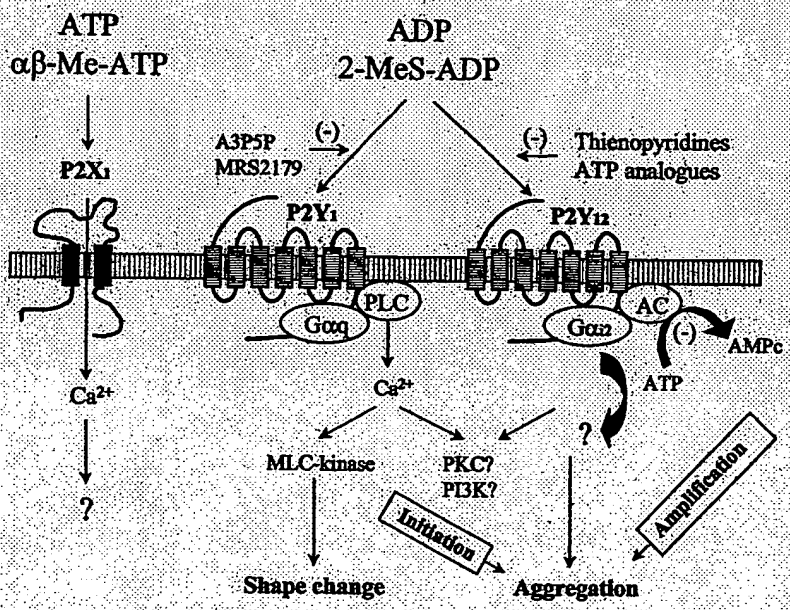

Figure 3 Three-receptor model of the ADP-induced platelet aggregation. Two ADP receptors are involved in ADP-induced platelet aggregation; the $P2Y_1$ receptor responsible for intracellular Ca^{2+} mobilization, shape change, and initiation of aggregation and $P2Y_{12}$, coupled to adenylyl cyclase inhibition, enhance $P2Y_1$-mediated platelet activation and secretion. $P2Y_{12}$ is the molecular target of the ADP-specific antiplatelet drug thienopyridines. Both receptors are required for normal platelet aggregation in response to ADP. $P2X_1$ is a receptor responsible for a rapid influx of calcium into the cytosol, but its role in the process of ADP-induced aggregation remains unknown.

change, release of calcium from internal pools, and IP metabolism in a $P2Y_1$-dependent manner, as shown by an inhibition by A3P5P and a lack of effect of the thienopyridines.[33,41–44] Recently, two different groups have generated $P2Y_1$-deficient mice.[45,46] These animals present strong defects in platelet functions, including shape change, cytosolic calcium elevation, and IP metabolism but also aggregation. This observation clearly indicates that the $P2Y_1$ pathway is activated during ADP-induced aggregation, as proposed previously by several authors.[41–43] It should also be noticed that purine-dependent calcium influx has been associated with the activation of another purinoreceptor present at the platelet surface: $P2X_1$.[47] The importance of this calcium channel in platelet activation remains controversial. We found that activation of this receptor by its major ligand, α-β methylene-ATP, did not potentiate nor attenuate ADP-induced shape change and aggregation.[47] Similar findings were also found in human platelets,[48] but a recent observation reports that platelets from a subject presenting a defect in the $P2X_1$ failed to aggregate after an ADP challenge.[49] Moreover, a recent article shows that $P2X_1$ is not activated by ADP.[50] Clopidogrel has been shown not to affect $P2X_1$ in rat platelets.[47]

Thienopyridine-Sensitive Platelet Purinoceptor: $P2Y_{12}$

The thienopyridine-sensitive receptor has been recently described by three groups including ourselves.[51–53] The first one,[51] using a "ligand fishing" approach, showed that the ligand of this receptor (named SP1999 in the article) was ADP and showed that it coupled with a chimeric Gqi protein in CHO cells. In the second article,[52] the authors explained that they found, by means of expression screening of a platelet library in *Xenopus* oocytes, a new P2Y receptor (tentatively named $P2Y_{12}$). Due to its sensitivity to ADP and coupling to a Gi protein, this receptor was proposed to be "the" receptor responsible for adenylyl cyclase downregulation and platelet aggregation.

In a recent work from our lab,[53] the encoding sequence of $P2Y_{12}$ was cloned and stably expressed in CHO cells. The kinetics of association of ^{33}P-2MeS-ADP to these $P2Y_{12}$-CHO cells was comparable with the kinetics described for platelets.[33] Equilibrium binding experiments revealed the presence of one class of binding sites exhibiting high affinity with an apparent equilibrium dissociation constant (KD) value of 0.61 ± 0.12 nM and a total number of binding sites (Bmax) corresponding to 477 ± 66 × 10^3 receptors per cell. The binding of ^{33}P-2MeS-ADP on $P2Y_{12}$-CHO cells was inhibited in the presence of increasing concentrations of unlabeled 2MeS-ADP. The concentration inhibiting 50% of the ^{33}P-2MeS-ADP binding (IC_{50}) was 1.12 ± 0.07 nM. A comparable competition was obtained with 2MeS-ATP (IC_{50} = 3.32 ± 0.62 nM). Other adenosyl-derived nucleotides (ADP, ATPβS, and ATP) totally displaced the binding of ^{33}P-2MeS-ADP to P2Y12-CHO cells but with a lower potency (IC_{50} = 2.5, 5.5, and 10.3 μM, respectively; Fig. 4A). UTP, a ligand of the $P2Y_2$ receptors, partly displaced the binding of ^{33}P-2MeS-ADP (IC_{50} >100 μM). pCMPS, the first described antagonist of platelet receptor,[54] inhibited the binding of ^{33}P-2MeS-ADP to $P2Y_{12}$-CHO cells (IC_{50} = 2.3 μM), whereas FSBA (an antagonist of aggregin)[55] and MRS2179 (an antagonist of $P2Y_1$)[56] did not show any effect (Fig. 4B). These results therefore indicate that the receptor expressed on $P2Y_{12}$-CHO cells dis-

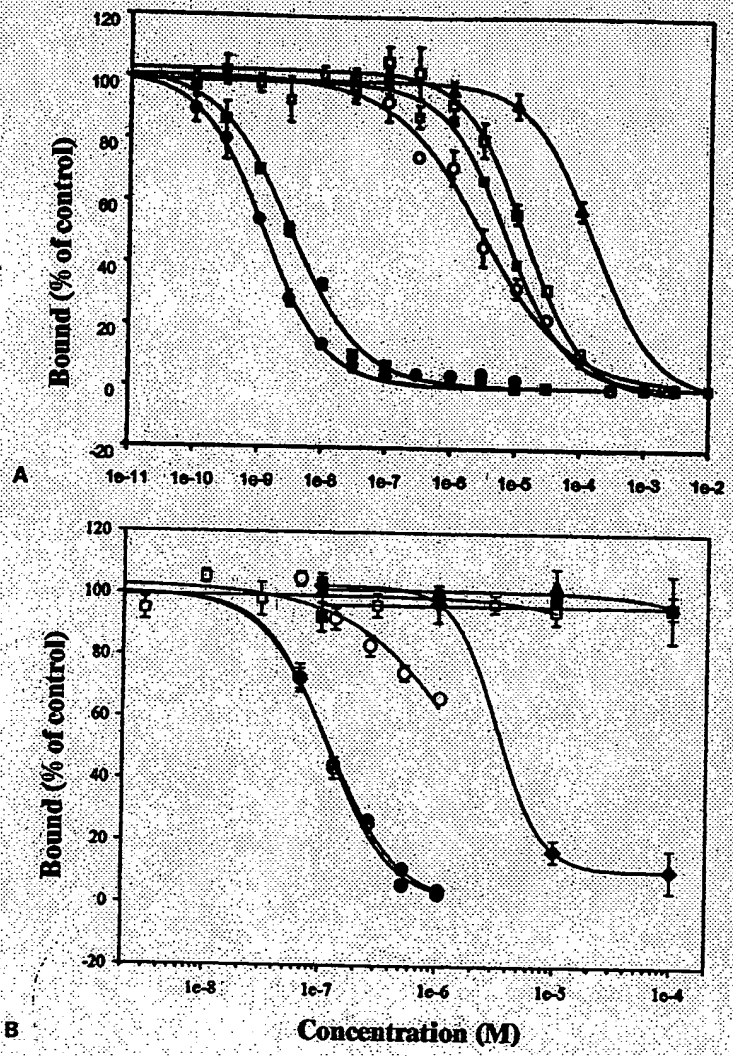

Figure 4 Effect of different compounds on the binding of ^{33}P-2MeS-ADP to $P2Y_{12}$-CHO cells. (A) Nucleotides; 2MeS-ADP (black circles), 2MeS-ATP (black squares), ADP (white circles), ATP (white squares), ATPγS (gray squares), and UTP (black triangles) were incubated for 10 minutes with $P2Y_{12}$-CHO cells and ^{33}P-2MeS-ADP (0.3 nM). (B) FSBA (black squares), MRS2179 (black triangles), and pCMPS (black diamonds) were incubated for 10 minutes with P2Y12-CHO cells and ^{33}P-2MeS-ADP (0.3 nM). The active metabolite of clopidogrel (black and gray circles), the R-metabolite (white circles), and clopidogrel (white squares) were preincubated with the cells for 1 hour at room temperature (RT). Cells incubated with the active metabolite were then either directly distributed (black circles) or previously centrifuged and resuspended in culture medium (gray circle). Then, the binding of ^{33}P-2MeS-ADP (0.3 nM) was measured.

plays a comparable biochemical profile as the $P2t_{AC}$ receptor described on platelets.[33,38]

Because clopidogrel is inactive in vitro, we tested the effect of the active metabolite of clopidogrel. This compound, purified from hepatic microsomes incubated with clopidogrel,[31] dose dependently affected the binding of ^{33}P-2MeS-ADP when incubated 1 hour prior to the ligand (IC_{50} = 100 nM). Moreover, if the cells were washed and resuspended in a metabolite-free medium, the effect persisted. This is indicative for an irreversible inhibition of the $P2Y_{12}$ receptor. The (inactive) R-enantiomer of clopidogrel only moderately decreased the ^{33}P-2MeS-ADP binding at high concentrations, and clopidogrel itself was ineffective in reducing the binding of ^{33}P-2MeS-ADP to these P2Y12-CHO cells (Fig. 4B). The basal cyclic AMP (cAMP) content of $P2Y_{12}$-expressing CHO cells was low and strongly increased when adenylyl cyclase was stimulated with forskolin. When the cells were costimulated with 0.1 and 1 nM 2MeS-ADP, the intracellular levels of cAMP dramatically decreased. As previously reported in platelets,[31] pretreatment of the cells with the active metabolite of clopidogrel prevented this inhibition (Table 3). These results therefore show that $P2Y_{12}$ is the previously called "platelet $P2t_{AC}$" receptor and that this receptor is antagonized by the active metabolite of clopidogrel.

CONCLUSION

Thienopyridines, by irreversibly antagonizing the recently described $P2Y_{12}$ receptor on platelets, provide long-lasting protection of platelets against ADP, a key mediator of thrombosis. The selectivity of thienopyridines with regard to platelet activation by ADP has allowed the significance of the latter to be evaluated in platelet physiology, pathophysiology, hemostasis, and thrombosis. These compounds have enabled the discovery of several ADP receptors present at the platelet surface and allowed several biochemical changes to be clearly attributed to one or the other of these platelet ADP receptors. Moreover, the existence of a congenital

Table 3 Effect of the Active Metabolite of Clopidogrel on Adenylyl Cyclase Downregulation in $P2Y_{12}$-CHO Cells

cAMP (pmol/10^6 cell)	Basal	Forskolin (10 μM)	Forskolin + 2MeS-ADP (0.1 nM)	Forskolin + 2MeS-ADP (1 nM)
CHO + vehicle	0.26 ± 0.06	2.63 ± 0.17	2.81 ± 0.24	2.50 ± 0.19
$P2Y_{12}$-CHO + vehicle	0.19 ± 0.05	1.57 ± 0.51	0.85 ± 0.21	0.62 ± 0.11
$P2Y_{12}$-CHO + active metabolite	0.44 ± 0.05	1.25 ± 0.31	2.55 ± 0.28	2.13 ± 0.54

Values are means ± SD ($n = 9$).

deficiency in ADP receptors, which duplicates the effects of a thienopyridine treatment, has enabled these observations to be confirmed. Beyond these observed cellular events, the effects on hemostasis and thrombosis demonstrate the in vivo importance of the $P2Y_{12}$ platelet purinoreceptor.

The specificity for such a relevant activation pathway, associated with a good tolerance profile of the drug, is predictive of the success of clopidogrel, which is today considered the gold standard in secondary prevention in atherosclerotic patients.

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A subclass of tumor-inhibitory monoclonal antibodies to ErbB-2/HER2 blocks crosstalk with growth factor receptors

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ErbB-2 is an orphan receptor that belongs to a family of tyrosine kinase receptors for either epidermal growth factor (EGF) or Neu differentiation factor (NDF/neuregulin). Because overexpression of the *erbB*-2 proto-oncogene is frequently associated with an aggressive clinical course of certain human adenocarcinomas, the encoded protein is an attractive target for immunotherapy. Indeed, certain monoclonal antibodies (mAbs) to ErbB-2 effectively inhibit tumor growth in animal models and in clinical trials, but the underlying mechanism is incompletely understood. To study this question, we generated a large battery of mAbs to ErbB-2, that were classified epitopically. Whereas most antibodies stimulated tyrosine phosphorylation of ErbB-2, their anti-tumor effect correlated with its accelerated endocytic degradation. One group of tumor-inhibitory mAbs (Class II mAbs) was elicited by the most antigenic site of ErbB-2, and inhibited *in trans* binding of NDF and EGF to their direct receptors. The inhibitory effect was due to acceleration of ligand dissociation, and it resulted in the reduction of the ability of ErbB-2 to transactivate the mitogenic signals of NDF and EGF. These results identify two potential mechanisms of antibody-induced therapy: acceleration of ErbB-2 endocytosis by homodimerization and blocking of heterodimerization between ErbB-2 and growth factor receptors.

Keywords: signal transduction; tyrosine kinase; oncogene; Neu differentiation factor; epidermal growth factor; adenocarcinoma

Introduction

The identification of tumor associated antigens (TAA) accessible on human cancer cells, hailed immunotherapeutic approaches relying on specific recognition of neoplasms (Hellstrom and Hellstrom, 1989). Extensive efforts have indeed been invested in examining the plausibility of anti-TAA monoclonal antibodies (mAbs) in the treatment of human malignancies proving promising in laboratory and clinic (Goldenberg, 1993). Protooncogene-encoded growth factor receptors are putative targets for such recognition-dependent therapy, due to their suggested role in pathological proliferation of cells (Aaronson, 1991). ErbB-2, a receptor-like tyrosine kinase, has been repeatedly implicated in cell transformation (Hynes and Stern, 1994; Stancovski *et al.*, 1994). Amplification of the corresponding gene and overexpression of the protein itself were observed in 20% to 30% of adenocarcinomas of the breast (King *et al.*, 1985; Slamon *et al.*, 1987, 1989), ovary (Slamon *et al.*, 1989), lung (Kern *et al.*, 1990) and stomach (Park *et al.*, 1989). Causative relationships between the cellular ErbB-2 content and the tumor's proliferative capacity and aggressiveness have been supported by different lines of evidence. When overexpressed in mouse fibroblasts, the human gene conferred a transformed phenotype *in vitro* and tumorigenesis *in vivo* (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987). Consistently, receptor overexpression is considered a predictor of poor survival and short time to relapse (Slamon *et al.*, 1987, 1989). Direct interference with the transforming potential of ErbB-2 has thus become a subject of great interest. Antibodies directed against the extracellular domain of either a mutated version of the rodent homolog of this receptor-like molecule, or against the human wild-type protein have been shown to confer inhibitory, as well as stimulatory, effects on tumor growth *in vivo* (Drebin *et al*, 1986; Hendly *et al.*, 1990; Hudziak *et al.*, 1989; Stancovski *et al.*, 1991). Moreover, a murine antibody capable of such growth inhibition has been recently humanized and tested in a phase II clinical trial, resulting in anti-tumor activity in patients with ErbB-2-overexpressing metastatic breast cancers (Baselga *et al.*, 1996).

Although the potential therapeutic use of anti-ErbB-2 mAbs is presently acknowledged and intensely examined, the molecular mechanisms underlying these effects are not well understood. Accelerated down-regulation of the receptor has been suggested to mediate antibody inhibition of cell transformation (Hudziak *et al.*, 1989; van Leeuwen *et al.*, 1990). However, the ability of mAbs to induce receptor internalization showed only partial correlation with anti-tumorigenic activity (Harwerth *et al.*, 1992; Hurwitz *et al.*, 1995). An obstacle in the understanding of mAb-mediated effects is the possibility that ErbB-2 has a direct ligand, that has not yet been completely characterized (Dougall *et al.*, 1994). Activation of receptor tyrosine kinases is dependent on receptor dimerization induced by the binding of specific ligands (Yarden and Schlessinger, 1987). However, ErbB-2 may participate in signal transduction even in the absence of a direct ligand, because it forms heterodimeric complexes with its family members, namely ErbB-1 (EGF receptor) and the two NDF/neuregulin receptors, ErbB-3 and ErbB-4 (Goldman *et al.*, 1990; Pinkas-Kramarski *et al.*, 1996; Riese *et al.*, 1995; Wada *et al.*, 1990). Hierarchical recognition of the dimerization partner offers ErbB-2 a key role in the determination and mediation of

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signaling and resultant cellular fate (Tzahar *et al.*, 1996). The importance of ErbB-2 as a transregulator expands in light of reported coexpression of ErbB family receptors in malignant cells (Gullick, 1990; Lemoine *et al.*, 1992), as well as by its ability to reconstitute the aberrant tyrosine kinase activity characteristic of ErbB-3 (Pinkas-Kramarski *et al.*, 1996; Riese *et al.*, 1995; Sliwkowski *et al.*, 1994). Heterodimer formation between ErbB-2 and ErbB-1 is responsible for synergistic growth signals in cells that co-overexpress the two receptors (Kokai *et al.*, 1989). A similar synergy was observed upon co-overexpression of ErbB-2 and ErbB-3 (Alimandi *et al.*, 1995; Wallasch *et al.*, 1995), probably due to the extremely high mitogenic potential of the corresponding receptor heterodimer (Pinkas-Kramarski *et al.*, 1996). Selective suppression of ErbB-2 expression at the cell surface by means of retention in the endoplasmic reticulum (Beerli *et al.*, 1994), demonstrated that this molecule can act as a shared signaling subunit of both EGF- and NDF-receptors (Graus-Porta *et al.*, 1995; Karunagaran *et al.*, 1996) that augments and prolongs signaling by deceleration of the rate of ligand dissociation (Karunagaran *et al.*, 1996).

The ability of ErbB-2 to serve as a pan ErbB auxiliary receptor subunit implies a versatility of mechanisms by which the receptor is involved in transformation. Hence, attempts to inhibit malignancies should consider the transacting potential of ErbB-2, in addition to its presumed ability to act through homodimer formation. Our study has addressed the possibility that mAbs directed against ErbB-2 might exert at least part of their tumor-inhibitory effects via interference with receptor-receptor interactions. A battery of antibodies directed against the extracellular domain of ErbB-2 has been generated and classified into groups according to specific epitope recognition. Several classes of tumor-inhibitory mAbs that accelerate cellular degradation of ErbB-2 were identified. Interestingly, one class of tumor-inhibitory antibodies partially reduced cellular binding of both NDF and EGF. Consistent with an ability to interfere with receptor crosstalk, these mAbs also reduced the trans-stimulatory effect of ErbB-2 on growth signals. We suggest that anti-ErbB-2 antibodies can inhibit cancer not only by impeding the homodimer-dependent activity, but also by blocking heterodimer formation and receptor crosstalk. This implies wider than currently accounted-for mechanisms that can be utilized for the designing of therapeutically efficient anti-ErbB-2 inhibitors. In addition, our results may be relevant to the mechanism by which EGF-like ligands recruit ErbB-2 into receptor heterodimers.

Results

Classification of anti-ErbB-2 monoclonal antibodies

To study the mechanistic basis of tumor inhibition by certain mAbs to ErbB-2, we extended our antibody repertoire by employing an exhaustive immunization protocol. Essentially, mice were immunized with a recombinant extracellular domain of the human protein and the resulting hybridomas were screened for antibody binding to a cell surface-expressed ErbB-2. A dozen of new mAbs was generated, and analysed together with a panel of five mAbs that we previously described (Stancovski *et al.*, 1991). The new mAbs were assayed for their ability to affect the tumorigenic growth in nude mice of the N87 human gastric carcinoma cell line overexpressing the ErbB-2 protein. Nine different mAbs, or saline as control, were injected intraperitoneally into groups of six mice, on days 3, 7 and 10 after tumor inoculation. Figure 1 depicts tumor progression in the presence of four representative mAbs. The tumorigenic growth of N87 cells was inhibited by 85%, 61% and 82% in nude mice injected with mAbs L26, L140 and L431, respectively. These values correspond to mean of inhibition measured at five time points throughout a period of 46 days post inoculation. Antibody L87 showed no effect on the growth of tumors *in vivo*, presumably due to its relatively low affinity (data not shown). Because previous studies suggested that different regions on the extracellular domain of ErbB-2 mediate mAb-dependent effects on tumor growth (Bacus *et al.*, 1992) we examined the dependency of tumor inhibition on specific immunogenic determinants by performing a reciprocal binding assay (Figure 2). Several mAbs were radiolabeled and their binding to N87 cells was determined in the presence of all other antibodies to enable classification of the mAbs. Thus, antibody L431 was efficiently displaced by the strong tumor-inhibitory mAb N12 (Stancovski *et al.*, 1991), but not by any other antibody (Figure 2A). Therefore, these mAbs were classified into the same group, denoted Class I. In a similar manner we could categorize mAbs L26, L96, and L288 into a second group, denoted Class II (Figure 2B) and mAb L140 into Class III, that comprises only a single antibody. Antibody L87 could be displaced by antibodies from several groups, although it could not fully displace its own binding

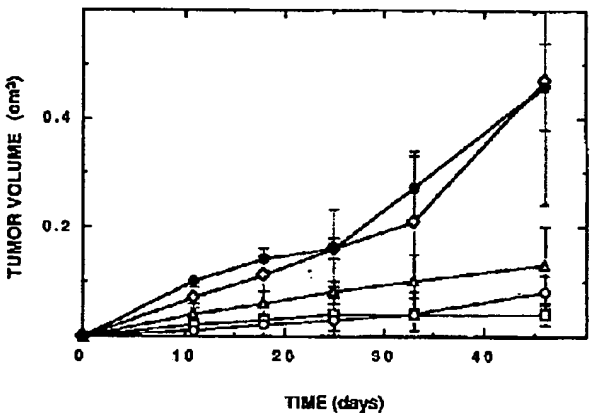

Figure 1 Inhibition of tumor growth by representative mAbs to ErbB-2. Athymic mice received a subcutaneous injection of 3×10^6 N87 human gastric cancer cells that overexpress ErbB-2. Three, 7 and 10 days later monoclonal antibodies (a total dose of 1 mg per animal) were injected intraperitoneally, and tumor volumes were measured at the end of the indicated time periods. Phosphate-buffered saline- (PBS-) injected mice were used for control (closed circles). The following mAbs were used: L26 (circles), L87 (rhombuses), L140 (triangles) and L431 (squares). Bars represent standard deviations for groups of five mice. The experiment was repeated three times with each of the mAbs and yielded similar results

(Figure 2D). This characteristic of mAb L87 is in accordance with its weak precipitating ability of the native ErbB-2 protein and is reinforced by the ability of mAb L87 to recognize the denatured protein (data not shown). The low affinity of mAb L87 to conformationally-intact ErbB-2 could underlie the observed pattern of displacement by a wide variety of mAbs. Several additional mAbs (e.g., L151, L242, L219, N28 and N29) were found to react with distinct antigenic determinants of ErbB-2, indicating multiplicity of antigenic sites, of which site II is apparently the most efficient. Anti-ErbB-2 antibody classification is summarized in Table 1.

Tumor inhibition correlates with mAb-induced receptor internalization but not with kinase activation

Upon binding of certain mAbs, ErbB-2 has been shown to undergo internalization (Drebin *et al.*, 1985) in a pathway shared by other growth factor receptors, when induced by ligands and antibodies (Sorkin and Waters, 1993). Several lines of evidence indicate a correlation between tumor-inhibitory activity of mAbs and their ability to accelerate ErbB-2 uptake and down-regulation (Hurwitz *et al.*, 1995; Tagliabue *et al.*, 1991). To test the applicability of such a correlation to the new inhibitory mAbs, we studied

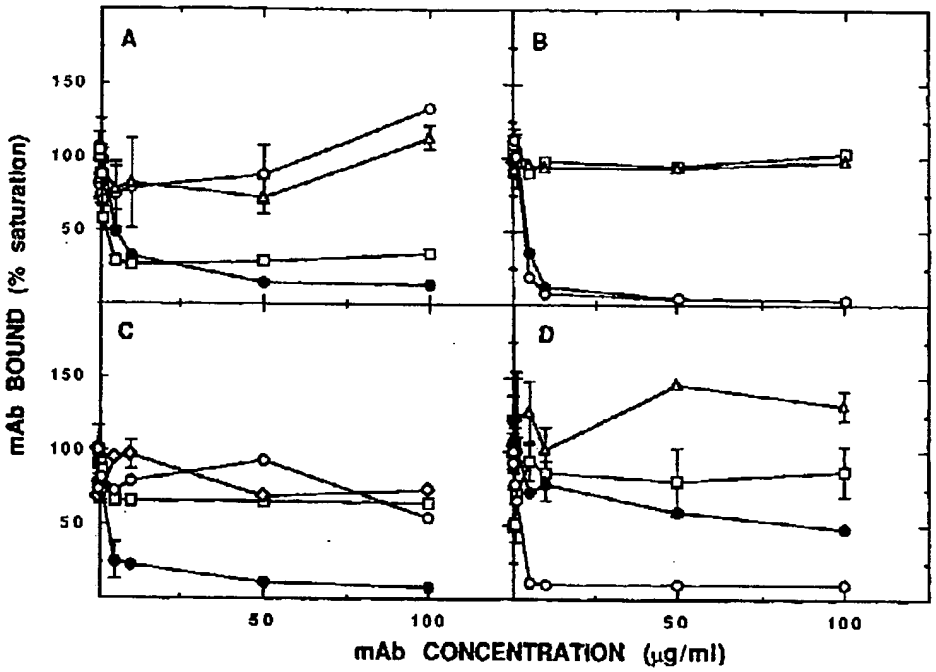

Figure 2 Analyses of competitive antibody binding to ErbB-2. The ability of unlabeled mAbs to displace a cell surface-bound ^{125}I-mAb was used as a measure for the degree of antigenic overlap. The following radiolabeled mAbs were used: L431 (A), L288 (B), L140 (C) and L87 (D). The labeled mAbs were added to the medium of N87 cells growing in 96-well culture dishes, in the presence of the same unlabeled antibody (closed circles). Alternatively, unlabeled mAbs representing the different classes were used: Class I (squares, N12 in A and C, L431 in B and D), II (circles, L26), III (triangles, L140) and IV (rhombuses, L87 in C). Following 1 h of incubation at 22°C, the monolayers of cells were washed three times with PBS and solubilized in an alkaline solution. The results are presented as the mean±s.d. of duplicates. The experiments were repeated thrice

Table 1

Antibody class	*Antibody*	*Tumor growth inhibition (%)*[a]	*Receptor internalization*[b]	*Receptor phosphorylation*[c]	*Ligand binding inhibition*[d] *EGF*	*NDF*
I	L431	82	+++	+	–	–
I	N12	86	+++	ND	ND	–
II	L26	85	++	±	+	+
II	L96	60	+	+	+	+
II	L288	74	++	+	±	ND
III	L87	–	–	–	–	
IV	L140	61	+	++		–
V	L151	40	–	+	ND	ND
VI	L219	49	ND	ND	ND	
VII	L242	61	+++	+		–
VIII	N28	40*		+	–	–
IX	N29	88	ND	+	–	–

[a]Average tumor volume inhibition of five time points, as percentage of control. [b]Receptor internalization was determined by cell surface protein labeling, as described in text and Figure 3 and its strength is expressed in correlation of the appearance of early (+), intermediate (±), and late (+++) degradation products. [c]Extent of induction of tyrosine phosphorylation of ErbB-2 protein in D2 cells was determined according to the assay in Figure 4. [d]Inhibition of EGF and NDF binding to T47D cells was determined as described in Figure 5. *Stimulatory antibody

their effect on the internalization of membrane-bound ErbB-2. The internalization assay used has not been previously applied to ErbB-2, and it included biotinylation of the surface-exposed protein, followed by exposure to the various mAbs. Molecules that underwent internalization escaped a subsequent digestion with pronase, that was applied extracellularily and were thus visualized by streptavidin detection. A 20 min-long incubation in the presence of mAbs from Class I (represented by L431) revealed a band of approximately 85 kDa, that represents a relatively late degradation product of ErbB-2 (Figure 3). A similar result was obtained upon incubation with the tumor-inhibitory mAb L242 (comprising a single-mAb group), whereas antibodies from Class II caused the appearance of the apparently early proteolytic product of 120 kDa, implying a slower degradation pathway. Internalization of ErbB-2 by mAb L140 (Class III), a moderate inhibitory mAb, resulted in both proteolytic products and a residual intact receptor (185 kDa protein band). Antibody L87 (Class VI), a non-inhibitory antibody, induced no detectable internalization of ErbB-2, an effect that was shared with the control IID2 antibody against α-fetoprotein. Because the size of the protein recovered by the internalization assay presumably reflects the rate of internalization, the results presented in Figure 3 suggest a dependency of tumor inhibition on the ability of the mAbs to internalize the receptor (Table 1). Of note, however, is the relatively slow endocytic processing that was induced by Class II mAbs (L26 and L288), implying that their strong anti-tumor effect depends on additional activities.

It has been previously reported that tumor-inhibitory effects of anti-ErbB-2 mAbs only partially correlate with modulations of the phosphotyrosine content of the receptor (Kumar *et al.*, 1991; Stancovski *et al.*, 1992). To examine the relationship between tumor inhibition and stimulation of ErbB-2 phosphorylation, we selected a model cellular system of 32D myeloid cells that ectopically express ErbB-2 in the absence of other ErbB proteins, thereby excluding transphosphorylation effects that widely occur within the ErbB family (Pinkas-Kramarski *et al.*, 1996). Lysates of antibody-treated cells, immunoblotted for the detection of proteins phosphorylated on tyrosine residues, demonstrated the dependency of ErbB-2 phosphorylation on mAb bivalency (Figure 4, upper panel). Tumor-inhibitory mAbs from both Class I and Class II (L431 and L26, respectively) caused a comparable extent of phosphorylation, that was absent when the monovalent antibody fragments (Fab) were used. Antibody L140, a moderate cancer effector, exerted maximal elevation of receptor phosphorylation, that was higher than the effect of the tumor-stimulatory mAb, N28 (Stancovski *et al.*, 1991). This suggests that the ability of antibodies to affect the extent of ErbB-2 phosphorylation reflects only their capacity to form ErbB-2 homodimers and as implied by Table 1 it may be independent of the long-term biological activity *in vivo*. Consistent with its low binding affinity, mAb L87 could not mediate a phosphorylation signal upon the receptor.

Class II mAbs inhibit ErbB-2 interactions with ErbB-familiy counterparts

The engagement of ErbB-2 in hetero-complexes with other ErbB-family tyrosine kinases, has been shown to augment both binding and signaling of EGF and NDF when associated with their respective receptors (Graus-Porta *et al.*, 1995; Karunagaran *et al.*, 1996; Kokai *et al.*, 1989; Sliwkowski *et al.*, 1994). To examine whether anti-ErbB-2 mAbs can interfere with heterodimer formation, we used ligand affinity as an indicator of ErbB-2 involvement in ligand binding complexes. This

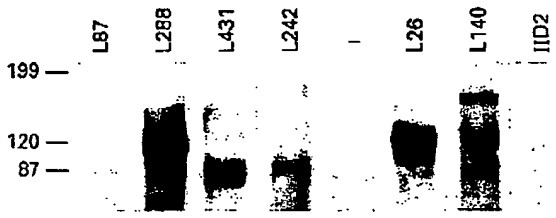

Figure 3 Effect of mAbs on degradation of ErbB-2. Cell surface proteins of confluent monolayers of N87 cells (10 cm dishes) were labeled with biotin as described under Materials and methods. The cells were then exposed for 30 min at 4°C to the indicated mAbs (at 40 μg/ml). The monolayers were thereafter incubated for 20 min at 37°C in order to allow receptor internalization and degradation. At the end of this incubation, the monolayers were transferred back to 4°C, treated for 30 min with pronase to digest surface-exposed proteins and cell lysates were prepared. ErbB-2 proteins that escaped hydrolysis by pronase were visualized by immunoprecipitation with the NCT antibody, that was followed by gel electrophoresis, transfer to nitrocellulose filter and detection with horseradish peroxidase-labeled streptavidin. Note that only internalized receptor is visualized. For control we incubated the cells in the absence of mAb (lane labeled -) or in the presence of an irrelevant mAb (antibody IID2). The locations of marker proteins are indicated in kilodaltons (kDa). The experiment was repeated twice

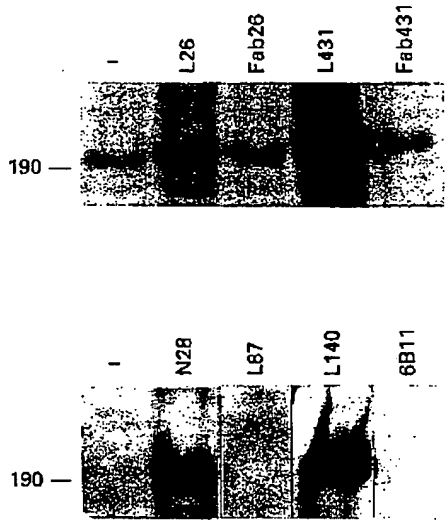

Figure 4 Antibody-induced stimulation of ErbB-2 phosphorylation on tyrosine residues. ErbB-2-expressing 32D cells (denoted D2 cells) were incubated for 15 min at 37°C with the indicated mAbs at 20 μg/ml, or with their respective monovalent fragments (Fab, 20 μg/ml). Whole cell lysates were then prepared and subjected to gel electrophoresis. The gel-resolved proteins were transferred to a nitrocellulose filter that was blotted with an antibody to phosphotyrosine and detected with a secondary antibody. Incubation in the absence of antibody (lane labeled) or with an isotope matched control mAb (antibody 6B11) were used for control. The location of a marker protein is indicated in kDa

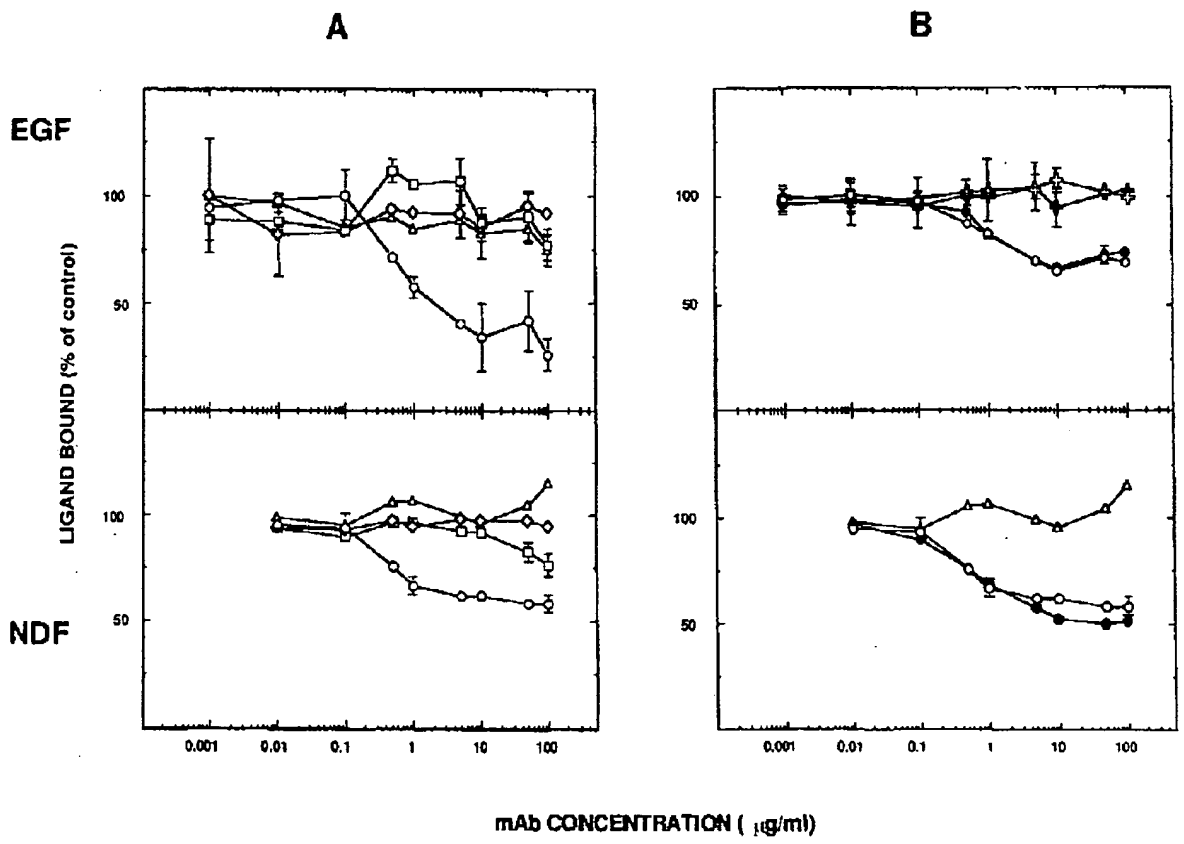

Figure 5 Effect of mAbs to ErbB-2 and their Fab fragments on receptor binding of NDF and EGF. (A) Monolayers of T47D cells growing in 48-well dishes were incubated for 2 h at 4°C with either ^{125}I-EGF or ^{125}I-NDF (each at 10 ng/ml) in the presence of increasing concentrations of the following mAbs that represent four different classes of mAbs: L431 (squares), L26 (circles), L140 (triangles), and L87 (rhombuses). Unbound radiolabeled ligand was then removed by washing and cell-associated radioactivity determined. (B) Experiments were conducted as in (A) to compare ligand binding in the presence of the Fab fragment of mAb L26 (closed circles) to the binding in the presence of the whole mAb (open circles). Antibody N28 (open crosses) and its Fab fragment (closed crosses) and antibody L140 (open triangles) served as controls. Each data point represents the average and standard deviation (bars) of duplicate determinations after subtraction of the non-specific ligand binding. The experiment was performed thrice

assay was performed on several cell lines, including N87 that expresses ErbB-1, ErbB-2 and ErbB-3 receptors and the human T47D breast cancer cell line, that expresses all four ErbB proteins. Figure 5A depicts the results of binding analyses of radiolabeled NDF and EGF, in the presence of representative mAbs directed against different ErbB-2 epitopes. It is important to note that none of the antibodies cross-reacted with other ErbB family members. Antibody L26, as well as other Class II mAbs, were able to displace up to 74% and 42% of cell-bound EGF and NDF, respectively. This phenomenon was not characteristic of mAbs capable of recognizing other receptor determinants (e.g., mAbs L431, L87 and L140, Figure 5A), suggesting that the epitope bound by mAbs from Class II is involved in the formation of heterodimers. This hypothesis was further supported by the inhibition of EGF and NDF binding to T47D cells by monovalent fragments of antibody L26 (Figure 5B). Fab fragments of L26 could inhibit the binding of both ligands, to an extent similar to that of the whole mAb, whereas the Fab of an antibody incapable of ligand binding inhibition (N28) could not. It is worth noting that all our ligand binding analyses were performed at 4°C, in order to exclude differences due to ErbB-2 internalization. Similar results were obtained with the N87 cell line (data not shown). To directly test the prediction that Class II mAbs inhibit ligand binding by interfering with the formation of ErbB-2-containing heterodimers, we covalently labeled each receptor with a radiolabeled ligand, and analysed coprecipitation of the affinity labeled receptor with ErbB-2. The results of this experiment, that was performed with N87 cells, are shown in Figure 6. Evidently, both monomeric and dimeric receptor species were precipitated by anti-ErbB-2 antibodies. However, the presence of mAb L26 during the affinity labeling reaction significantly reduced coprecipitation with ErbB-2. The effect was larger with EGF than with NDF, consistent with the results of the ligand displacement assay (Figure 5A), and it was not induced by a control non-relevant antibody (6B11). However, all mAbs to ErbB-2 slightly reduced the efficiency of affinity labeling, especially with EGF, probably due to aspecific masking of primary amino groups.

To further study the mechanism underlying antibody-induced inhibition of ligand binding we measured the effect of mAb L26 on the affinity of EGF and NDF to their receptors (Figure 7A). Scatchard analyses revealed a minimal effect on the number of binding

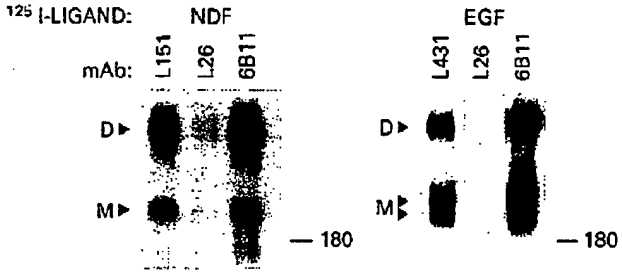

Figure 6 The effect of mAbs on affinity labeling and co-immunoprecipitation of NDF- and EGF-receptors with ErbB-2. Monolayers of confluent N87 cells were incubated for 2.5 h with 10 ng/ml of ^{125}I-labeled NDF-β1$_{177\ 246}$ or EGF, in the presence of mAb L26 (5 and 25 μg/ml, left and right panels respectively), L151 (5 μg/ml), or L431 (25 μg/ml), as indicated. A control mAb, 6B11, was also added (5 and 25 μg/ml, left and right panels respectively). The cell monolayers were washed with PBS and then subjected to affinity labeling for 25 min with BS^1 (1 mM), followed by cell lysis. After clearance of cell debris, the detergent-solubilized lysates were subjected to separate immunoprecipitation reactions with NCT, a rabbit polyclonal antiserum directed to the C-terminus of the ErbB-2 protein. Immunocomplexes were resolved by gel electrophoresis on a 6.5% acrylamide gel followed by autoradiography. Note that monomeric receptor is coprecipitated representing ligand bound receptor that is probably involved in heterodimers

sites, but a significant reduction in ligand affinities upon co-incubation with a Class II mAb: threefold for EGF and twofold for NDF. Because ErbB-2-containing heterodimers are characterized by a relatively slow rate of ligand association (Karunagaran *et al.*, 1996), we tested the prediction that an acceleration in ligand dissociation was responsible for the observed inhibitory effect of mAb L26. The kinetics of EGF and NDF release were determined in the presence or absence of the L26 mAb. Evidently, the mAb significantly increased the rates of dissociation of both ligands from their cellular binding sites (Figure 7B). Taken together, the results of ligand binding analyses imply that mAbs belonging to Class II are capable of interfering with the stability of dimers formed between ErbB-2 and its family members, namely EGF- and NDF-receptors.

Class II mAbs inhibit transactivation of growth-regulatory signals by ErbB-2

Prolonged binding of NDF and EGF achieved by the presence of ErbB-2 is thought to augment growth-regulatory signals. Indeed, ErbB-2 has the ability to potentiate the proliferative effect of EGF and to

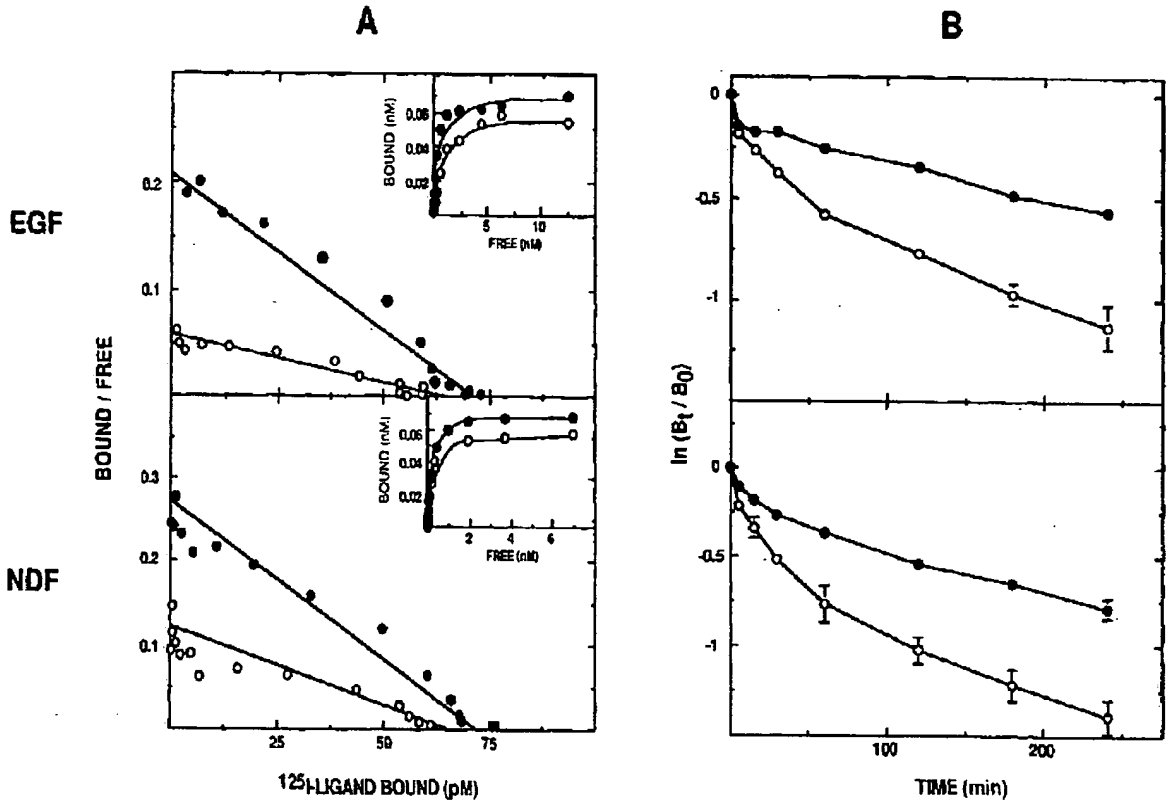

Figure 7 Demonstration of mAb-induced changes in NDF and EGF binding. (A) Reduction of ligand affinity. Monolayers of T47D cells were incubated for 2 h at 4°C with increasing concentrations of ^{125}I-NDF or ^{125}I-EGF in the presence (open circles) or absence (closed circles) of mAb L26 (50 μg/ml). Unbound ligand removal was followed by cell lysis and radioactivity monitoring. Non-specific ligand binding was determined in the presence of a 100-fold excess of the unlabeled ligand, and was subtracted from the total amount of ligand that bound at each concentration. The results are presented as Scatchard plots and as saturation curves (insets). Each data point represents an average of a duplicate, and the whole experiment was repeated thrice. (B) Acceleration of ligand dissociation. T47D cells were first incubated for 2 h at 4°C with radiolabeled EGF or NDF (each at 20 ng/ml) as indicated. Thereafter, the ligands were removed and the cells incubated with an unlabeled ligand (200 ng/ml) and in the presence (open circles) or absence (closed circles) of mAb L26 (20 μg/ml). Cell-bound as well as released radioactivity were then followed as a function of time of incubation at 4°C. The results are expressed as the ratio between the amount of ligand that was bound at time t (B_t) and the initially bound ligand (B_0) and they represent the average and standard deviation of duplicates. The experiments were repeated twice

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